Peer-Reviewed Articles
Theme 2 - Clonal Evolution and Tumor Cell Kinetics

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Research Project • Clonal Evolution and Topographic Tumor Heterogeneity •

PRENEOPLASTIC LESION

NEOPLASTIC LESION

First Mutation

Irreversible Neoplastic Change

Clinically Detectable Neoplasm

Cooperative Mutational Events

No Evidence of Genetic Damage

Evidence of Genetic Damage, Consistent with Clonal Expansion

Clonal Proliferation

Clone Selection

Genetic Changes

T C

T C
Clonal Evolution and Tumor Cell Kinetics


Salvador J. Diaz-Cano,* † Manuel de Miguel, ‡ Alfredo Blanes, § Robert Tashjian,* Hugo Galera, ‡ and Hubert J. Wolfe*

Clonal Patterns in Phaeochromocytomas and MEN-2A Adrenal Medullary Hyperplasias: Histological and Kinetic Correlates. J Pathol 2000;192:221-228

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Kinetic profiles of intraepithelial and invasive prostatic neoplasias: the key role of down-regulated apoptosis in tumor progression. Virchows Arch 2000;436:413-420

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ACTIVATING POINT MUTATIONS IN CYCLIN-DEPENDENT KINASE 4 ARE NOT SEEN IN SPORADIC PITUITARY ADENOMAS, INSULINOMAS OR LEYDIG CELL TUMOURS. J ENDOCRINOL. 2003 AUG;178(2):301-10.


HASHIMOTO'S THYROIDITIS SHARES FEATURES WITH EARLY PAPILLARY THYROID CARCINOMA. HISTOPATHOLOGY. 2002 OCT;41(4):357-62. REVIEW.

Arif S, Blanes A, Diaz-Cano SJ.

KINETIC PROFILES OF INTRAEPITHELIAL AND INVASIVE PROSTATIC NEOPLASIAS: THE KEY ROLE OF DOWN-REGULATED APOPTOSIS IN TUMOR PROGRESSION. Virchows Arch 2000;436:413-420

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TUMOR SCREENING AND BIOLOGY IN MALIGNANT MELANOMAS. ARCH DERMATOL. 2000 JUL;136(7):934-5.

Pozo L, Diaz-Cano SJ.
Clonality as Expression of Distinctive Cell Kinetics Patterns in Nodular Hyperplasias and Adenomas of the Adrenal Cortex

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Although histopathologic criteria for adrenal cortical nodular hyperplasias (ACNHs) and adenomas (ACAs) have been developed, their kinetics and clonality are virtually unknown. We studied 20 ACNHs and 25 ACAs (based on World Health Organization criteria) from 45 females. Representative samples were histologically evaluated, and the methylation pattern of the androgen receptor alleles was analyzed on microdissected samples. Consecutive sections were selected for slide cytometry, flow cytometry, and in situ end labeling (ISEL). Apoptosis was studied by flow cytometry (nuclear area/DNA content plotter analysis) and by ISEL. Appropriate tissue controls were run in every case. Polyclonal gel patterns were revealed in 14/18 informative ACNHs and in 3/22 informative ACAs, whereas monoclonal gel patterns were observed in 4/18 ACNHs and 19/22 ACAs. Overlapping proliferation rates (PRs) were observed in both clonal groups, and apoptosis was detected only in G0/G1 cells, especially in monoclonal ACNHs (3/4; 75%) and in polyclonal ACNHs (2/3; 67%). Significantly higher PRs were observed in ACNHs with polyclonal patterns and G0/G1 apoptosis and in ACAs regardless of clonality pattern and presence of G0/G1 apoptosis. All except one ACNH (19/20; 95%) and 15/25 ACAs (60%) showed diploid DNA content, whereas the remaining cases were hyperdiploid. A direct correlation between PR and ISEL was observed in polyclonal lesions (PR = 29.32 ISEL – 1.93), whereas the correlation was inverse for monoclonal lesions (PR = −9.13 ISEL + 21.57). We concluded that only simultaneous downregulated apoptosis and high proliferation result in selective kinetic advantage, dominant clone expansion, and unbalanced methylation patterns of androgen receptor alleles in ACNHs and ACAs. (Am J Pathol 2000, 156:311–319)

Neoplasms result from the progressive and convergent selection of cell populations, but several factors should be considered. On one hand, selection will determine tumor progression and cellular heterogeneity. On the other hand, cellular selection must be related to cell kinetics process.1,2 All genetic abnormalities seen in tumors should be fixed on the transformed cell before ending in a fully established malignancy. These genetic changes must be cooperative and resistant to the cellular repair systems, and they must not activate the apoptosis pathway. This process determines a complex network in which a potential genetic marker will be useful when associated with kinetic advantages responsible for cellular outgrowths. In general, proliferation markers are very important for the distinction between benign and malignant endocrine neoplasms and for the analysis of tumor progression.3,4 Actually, the most important parameter for the diagnosis of adrenal cortical neoplasms is the mitotic figure counting. However, any proliferative advantage resulting in dominant growth could be caused by either a high proliferation rate or abnormally low apoptotic indices. The key kinetic factor is the imbalance between cell proliferation and cell loss resulting in tissue overgrowth.5,6 In this regard, variable results have been reported for adrenal cortical tumors,7 and no reference is available for nodular hyperplasias.

Clonal origin is still the hallmark of neoplasms and strongly indicates acquired somatic mutations that give survival advantage to a cell population.8 The acquisition of additional genetic deletions in certain histological areas favors a molecular progression.9 However, the molecular events in the transformation pathways are not completely understood and, in many instances, remain essentially unknown. Under those circumstances, clonality assays based on the analysis of X-chromosome inac-
tivation in females represent the best molecular option, although this option is restricted to a subset of informative females. These assays are based on the ability to distinguish the paternally inherited X chromosome from the maternally inherited one, and they do not rely on the presence of any tumor-related genetic alteration.\(^9,10\) Monoclonal patterns suggest neoplasia but are not diagnostic of neoplasia. Yet, clonality offers a better understanding of tumors if it is combined with kinetic features (proliferation and apoptosis). No previous study has focused attention on those parameters (clonality and cell kinetics) in benign proliferative lesions of the adrenal cortex, in which the distinction between nodular hyperplasias and adenomas is sometimes controversial.\(^11\)

This study addressed the clonal evaluation of adrenal cortical nodular hyperplasias (ACNHs) and adenomas (ACAs), based on an analysis of methylation patterns of androgen receptor alleles, using microdissected tissue samples. The kinetic features of these lesions were also analyzed by means of proliferation and apoptotic markers.

**Materials and Methods**

**Case Selection and Sampling**

Consecutive adrenal cortical proliferative lesions (64) were selected and histologically evaluated.\(^12\) Of these lesions, 53 were detected in female patients, including eight malignant tumors. ACNHs (20) and ACAs (25) were studied and classified by World Health Organization criteria,\(^11\) although evidence of metastases was the main criterion for malignancy and case exclusion. The mean follow-up time in this series was 135 months.

All surgical specimens were serially sectioned and embedded for routine histopathologic diagnosis (at least 1 block/cm). The most cellular areas from the biggest nodule in each case of ACNH and from every ACA were screened and selected for further analysis. The same areas were used in each analysis; hematoxylin and eosin (H&E)-stained sections taken before and after the specimen samples were used to check the cellular composition of each sample.

**X-Chromosome Inactivation Assay for Clonality Analysis**

Two 20-μm unstained paraffin sections were used for microdissection under microscopic control. Adrenal cortical cells and controls (histologically normal adrenal cortex, adrenal medulla, and periadrenal soft tissue from the same slide) underwent DNA extraction. At least two separate areas of 0.25 mm\(^2\), containing about 100 target cells each, were harvested from both peripheral and internal areas of the biggest nodule in ACNH or ACA.

The samples were dewaxed with xylene, cleared with absolute ethanol, and digested with proteinase K; DNA was extracted using a modified phenol-chloroform protocol, as previously described.\(^13\) All samples were divided for restriction endonuclease digestion with *Hha*1 (New England Biolabs, Beverly, MA). Half of each sample underwent enzymatic digestion (0.8 U/μl), while the remaining half was kept as undigested control. The undigested samples were processed like the digested ones but excluding *Hha*1 in the reaction mixture. 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 glyco- gen) at 37°C for 4 to 16 hours. A mimicker (0.3 μg of double-stranded and Xhol-linearized *φX174-RII* phage; Life Technologies, Inc., Gaithersburg, MD) was included in each reaction mixture. Complete digestion was checked by gel electrophoresis; incompletely digested samples were phenol chloroform-purified and redigested with higher *Hha*1 concentrations.

*Hha*1 was then inactivated by phenol-chloroform extraction as previously described.\(^13\) 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 MgCl\(_2\), 100 μg/ml bovine serum albumin). Both *Hha*1-digested DNA and undigested DNA were then used for PCR amplification of the CAG repeat in the first exon of the human androgen receptor gene (*HUMARA*). The PCR products also included a DNA sequence recognized by *Hha*1, which is consistently methylated in the inactive *HUMARA* allele only.\(^14,16\) Primers and PCR cycling conditions were designed as previously described.\(^1,15,17\) The reactions were run in duplicate and optimized for a 10-μl reaction in a Perkin-Elmer thermal cycler model 480 (Perkin-Elmer, Norwalk, CT).

The whole PCR volume was electrophoresed into 0.75-mm-thick 8% nondenaturing polyacrylamide gel at 5 V/cm until a xylene cyanol band was located within the bottom inch of the gel. After fixation with 7% acetic acid (5 minutes), the gels were dried under vacuum (80°C, 40 minutes) and put inside a developing cassette containing one intensifying screen and prefilled filters (Kodak XAR) facing the intensifying screen (16–48 hours, −70°C). The autoradiograms were developed using an automated processor Kodak-X-Omat 100 (Kodak Co., Rochester, NY).

Interpretation and inclusion criteria in each sample were as previously reported.\(^1\) Allelic imbalance was densitometrically evaluated (EC model 910 optical densitometer, EC Apparatus Corp., St. Petersburg, FL), and evidence of monoclonal proliferation was considered to be allele ratios \(\geq 4:1\) with the normalized *Hha*1-digested samples. Sample normalization was done in relation to the corresponding undigested sample and tissue controls. Only informative cases (two different alleles in *Hha*1-undigested and *Hha*1-digested samples) were included in the final analysis.\(^8,15,17\)

**Slide Cytometric Analysis of DNA Content**

Feulgen-stained sections were used for DNA quantification.\(^18\) The densitometric evaluation was performed with the cell analysis system model 200 and the quantitative
DNA analysis package as software (Becton Dickinson). At least 200 nuclei were measured in every case, beginning in the most cellular area, until completion in consecutive microscopic high-power fields. Only complete, non-overlapping, and focused nuclei were quantified in each field.

External staining calibration was carried out with complete rat hepatocytes (Becton-Dickinson; one slide per staining holder) to normalize the internal controls; the latter included both lymphocytes and adrenal cortical cells from histologically normal areas present in the same tissue section. 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 $G_2 + M$ cells). 19

Proliferation rate ($PR = S$-phase + $G_2$-phase + M-phase fractions) was calculated from the DNA histogram by subtracting the number of cells within $G_0/G_1$ limits from the total number of measured cells. The values were compared with the total cell number and expressed as percentages. 19

Nuclear DNA Quantification by Flow Cytometry

Serial 50-μm-thick sections were microdissected, and nuclear preparations were stained with propidium iodine after RNase A digestion to study DNA ploidy (by the technique of Hedley et al). 20 DNA quantification parameters included DNA indices and PRs as previously described. 19 The scatter analysis of nuclear area and DNA content allowed the identification of apoptotic cells in each cell cycle phase (low nuclear area for a given DNA content allowed the identification of apoptotic cells in flow cytometry. Variables showing normal distribution were analyzed using a two-tailed Student's t-test, whereas analyses of variance were used for variables with nonparametric distribution. Normal distribution was previously tested by the Kolmogorov-Smirnov test. The results were considered statistically significant if $P < 0.05$. Regression analyses were also performed to test the correlation between proliferation and apoptosis markers in both polyclonal and monoclonal lesions.

Statistical Analysis of Quantitative Variables

The results of quantitative variables were compared by diagnostic groups (ACNH versus ACA), clonality pattern (polyclonal versus monoclonal), and the presence of $G_0/G_1$ apoptotic cells in flow cytometry. Variables showing normal distribution were analyzed using a two-tailed Student's t-test, whereas analyses of variance were used for variables with nonparametric distribution. Normal distribution was previously tested by the Kolmogorov-Smirnov test. The results were considered statistically significant if $P < 0.05$. Regression analyses were also performed to test the correlation between proliferation and apoptosis markers in both polyclonal and monoclonal lesions.

Results

The analysis of the methylation pattern of androgen receptor alleles showed polyclonal patterns in 14/18 (78%) informative ACNHs and in 3/22 (14%) informative ACAs, whereas patterns were monoclonal in 4/18 (22%) informative ACNHs and in 19/22 (86%) informative ACAs (Table 1, Figure 1). Females showing unbalanced methylation of androgen receptor alleles in histologically normal adrenal cortex were considered noninformative and excluded from further analyses (two ACNHs and three ACAs). Consistent methylation patterns were detected in both peripheral and internal samples from the same cortical nodule or tumor. However, two samples from internal areas of ACAs were contaminated with host stromal cells and showed pseudopolyclonal gel patterns.

The combined analysis of nuclear area and DNA content revealed features of apoptosis (reduced nuclear size for a given DNA content) only in $G_0/G_1$ cells (Table 2, Figure 2). An inverse relationship between the detection of $G_0/G_1$ apoptotic cells and the clonal pattern was also
Table 1. Results of Proliferation, Apoptosis, and Clonality in ACNHs and ACAs

<table>
<thead>
<tr>
<th>Case</th>
<th>PR by slide cytom. (%)</th>
<th>PR by flow cytom. (%)</th>
<th>DNA ploidy (DNA index)</th>
<th>ISEL (%)</th>
<th>G0/G1 apoptosis</th>
<th>Methylation of AR alleles</th>
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<td>ACNH-1</td>
<td>31.61</td>
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</tbody>
</table>

Abbreviations: ACNH, adrenal cortical nodular hyperplasia; ACA, adrenal cortical adenoma; PR, proliferation rate; ISEL, in situ end labeling; AR, androgen receptor; DIP, diploid; ANEUP, aneuploid; POLYP, Polyploid; cytom., cytometry; Noninform., noninformative.

*Small proportion of hyperdiploid G0/G1 cells (DNA index = 1.48) also identified.
†Small proportion of hyperdiploid G0/G1 cells (DNA index = 1.60) also identified.

revealed: G0/G1 apoptotic cells were detected in 3/4 (75%) of monoclonal ACNHs and 2/3 (67%) of polyclonal ACAs, using flow cytometric criteria.

Variable proliferation rates defined both polyclonal and monoclonal ACNHs. However, a direct and linear correlation was observed between proliferation and apoptosis in polyclonal ACNHs, although an inverse and linear correlation defined monoclonal ACNHs (Tables 1 and 2, Figure 3). Proliferation rates were significantly higher in polyclonal ACNHs with G0/G1 apoptosis than in polyclonal ACNHs with no G0/G1 apoptosis (P = 0.005) and were higher in monoclonal ACNHs with no G0/G1 apoptosis than in monoclonal ACNHs with G0/G1 apoptosis (Table 2, Figure 4; P = 0.009). Likewise, significantly higher ISEL indices were observed in monoclonal ACNHs (0.80 ± 0.14) than in polyclonal ACNHs (0.54 ± 0.16; P = 0.03). The flow cytometric detection of G0/G1 apoptotic cells correlated with the in situ detection; higher ISELS were observed in cases with G0/G1 apoptotic cells (ISEL index = 0.86 ± 0.05) than in those without (ISEL index = 0.49 ± 0.24; P = 0.05).

Polyclonal and monoclonal ACAs also showed overlapping and variable proliferation rates, but inversely and linearly correlated with ISEL indices in the monoclonal ACA group (Figure 3). Monoclonal ACAs with no G0/G1 apoptotic cells showed significantly higher proliferation rates than monoclonal ACAs with G0/G1 apoptotic cells (P = 0.02; Table 2, Figure 4). Again, the ISEL indices...
directly correlated with the flow cytometric detection of G0/G1 apoptotic cells (Table 1, Figure 2); ACAs showing G0/G1 apoptotic cells displayed higher ISEL indices than ACAs without G0/G1 apoptotic cells (1.77 ± 0.58 versus 0.80 ± 0.50; P = 0.03). On the other hand, highly variable ISEL indices were observed in both polyclonal ACAs (1.33 ± 0.64) and monoclonal ACAs (0.92 ± 0.33), with no significant differences (P = 0.14). No significant differences appeared for polyclonal ACAs.

All except one (19/20) ACNHs were diploid, whereas 15/25 (60%) ACAs showed diploid DNA content. One additional ACA was classified as polyploid, and the remaining cases revealed hyperdiploid DNA contents (Table 1). No significant differences were found for both proliferation and ISEL indices after DNA-ploidy stratification, although hyperdiploid ACAs tended to show greater scores than diploid ACAs (Table 3). Hyperdiploid lesions from informative patients preferentially revealed monoclonal patterns (6/7, 86%, including 5/6 ACAs and 1/1 ACNHs) and G0/G1 apoptotic cells in 3/7 cases (43%, 1 monoclonal ACA, 1 polyclonal ACA, and 1 monoclonal ACNH). Proliferation rates were higher in ACNHs than in ACAs, after stratification by clonality pattern and flow cytometric detection of G0/G1 apoptotic cells (Table 2). The homogeneous DNA-ploidy distribution in ACNH precluded additional analyses in this group.

Discussion

This study represents the first report on combined features of clonality and cell kinetics in ACNHs and ACAs. The distinctive correlation between apoptosis and proliferation and the heterogeneous clonal patterns revealed by ACNHs and ACAs were the most important findings.

Regarding the correlation between apoptosis and proliferation, polyclonal lesions (14/17 ACNHs, 82%) demonstrated increasing apoptosis as a counterpoise to rising proliferative rates. Monoclonal lesions (19/23 ACAs, 83%), however, had progressively lower apoptotic rates as proliferation increased. That inverted relationship between apoptosis and proliferation in monoclonal adrenal cortical lesions also provides a functional basis for clonal selection and segregates ACNHs from neoplastic ACAs.

Cell kinetics represent the basic mechanisms leading to clonal expansions and tumor growths.2,6 The correlation between proliferation and apoptosis provides rules for cellular selection, ie, clonal expansion or regression. Down-regulated apoptosis (as revealed by ISEL) has been reported in intraepithelial neoplasms of different locations and would allow both survival and replication of genetically damaged cells, giving rise to mutation accumulation in those cells.26 A maintained cell proliferation would transmit those genetic changes into descendant cells, and a relatively blocked apoptotic process would allow genetically damaged cells to complete the replication cycle, ending in mutation accumulation and tumor promotion.27 Inversely related proliferation and apoptosis in monoclonal lesions (Figure 3) would then contribute to clonal progression and would represent additional evidence of the cell cycle dysregulation found in neoplasms.6,7,28 The opposite scenario would lead to clone

Table 2. Comparative Results of Proliferation Indices by Apoptosis and Clonality Patterns

<table>
<thead>
<tr>
<th>Clonality pattern</th>
<th>Proliferation rate slide cytometry (Av ± SD)</th>
<th>Proliferation rate flow cytometry (Av ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ G0/G1 apoptosis</td>
<td>− G0/G1 apoptosis</td>
</tr>
<tr>
<td>Polyclonal ACNH</td>
<td>32.09 ± 0.68</td>
<td>13.84 ± 9.68</td>
</tr>
<tr>
<td>Polyclonal ACA</td>
<td>10.97 ± 1.90</td>
<td>11.73</td>
</tr>
<tr>
<td>Monoclonal ACA</td>
<td>15.21 ± 6.46</td>
<td>24.46 ± 10.54</td>
</tr>
</tbody>
</table>

ACNH, adrenal cortical nodular hyperplasia; ACA, adrenal cortical adenoma; Av, average; SD, standard deviation.
regression. A few monoclonal cases showed diploid DNA content with relatively low proliferation and high apoptosis (3/4 monoclonal ACNHs and 4/18 monoclonal ACAs). That index combination provides a negative selection factor that would determine slow tumor growth. Those cases showed more frequently regressive changes and atypical nuclear features (size variation, contour irregularity, and hyperchromatism). Only 2 of 17 polyclonal lesions (one DNA-diploid and one DNA-aneuploid lesion) showed similar kinetic features (Figure 3) and ACA histopathology. On the other hand, only one monoclonal ACNH displayed kinetic features in the polyclonal domain (Figure 3), with higher proliferation than polyclonal ACNH but with a similar ISEL index. Histologically, this monoclonal ACNH showed a coexistent ACA, supporting the close correlation of proliferation, apoptosis, and clonality in these neoplasms.

ACNH and ACA showed a heterogeneous clonal profile as previously reported with polyclonal lesions predominating in ACNHs (78% of informative cases) and monoclonal lesions predominating in ACAs (86% of informative cases). Endocrine hyperplasias have shown polyclonal patterns in hereditary and nonhereditary hyperparathyroidism or multinodular goiters. Most parathyroid or thyroid adenomas have been found to be monoclonal. Those findings support the concept of multistep tumorigenesis and Knudson’s hypothesis. However, monoclonal hyperplasias (parathyroid, in multiple endocrine neoplasia type 1 (MEN-1) and uremic patients, and multinodular goiters) and polyclonal adenomas (parathyroid and thyroid) have also been reported. Therefore, it has been concluded that clonality assay itself is of limited utility in differentiating hyperplastic from neoplastic conditions.

Of our ACAs, 14% were polyclonal, a proportion similar to those reported in parathyroid adenomas, thyroid adenomas, and ACAs. There are three possible explanations for polyclonal patterns in neoplasms.

1) The selective methylation of an inactive X chromosome is normally demonstrated by using methylation-
sensitive restriction endonucleases. Both incomplete endonuclease digestion and aberrant hypermethylation (due to tumor progression or abnormal imprinting) would result in false polyclonal patterns in monoclonal tissues. Only informative cases in clonality analysis were included in this regression analysis.

2) Any significant contamination as a potential cause of pseudopolyclonal patterns could be excluded by both careful microdissections under microscopic control and multiple sampling from a single nodule or tumor. ACAs tend to show myxoid stromal change in the central areas. Besides its diagnostic utility, that myxoid stromal change can contaminate the samples, giving false polyclonal patterns. It is normally associated with vascular ectasia and hemorrhage, especially in the internal ACA area, and should express the requirements of tumor growth. Preliminary morphometric data (unpublished results) have shown bigger vascular areas of sinusoid-like structures in ACAs than in ACNHs. 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 a key element of epithelial cell growth either by secretion of stimulatory factors or lack of an inhibitory factor in experimental thyroid nodules.

3) Finally, true polyclonal proliferations can explain those results, but additional markers would be required.

Table 3. Proliferation and ISEL Indices by DNA Content in ACAs

<table>
<thead>
<tr>
<th>ACA DNA content</th>
<th>Proliferation rate (Av ± SD, %)</th>
<th>ISEL (Av ± SD, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid ACA</td>
<td>12.15 ± 5.84</td>
<td>1.06 ± 0.59</td>
</tr>
<tr>
<td>Aneuploid ACA</td>
<td>17.88 ± 6.80</td>
<td>1.77 ± 0.58</td>
</tr>
</tbody>
</table>

ACA, adrenal cortical adenoma; ISEL, in situ end labeling; Av, average; SD, standard deviation.
Similarly, monoclonal patterns can be explained in non-neoplastic conditions. Proliferations of unselected cells from a polymorphic tissue show polyclonal patterns, owing to the mosaic distribution in the random distribution of X-chromosome inactivation.\textsuperscript{8,39–41} However, the relative sizes of cell groups sharing the same inactivated X chromosome (patch size concept or contiguous cellular regions of the same lineage) and embryological reasons determine the clonal pattern. Any kinetic advantage in small cell groups sharing the same inactivated X chromosome would result in their preferential growth, thus yielding an overall monoclonal pattern\textsuperscript{42} even in early stages.\textsuperscript{39,44} These selective growth advantages would also explain monoclonal proliferations in both early neoplasms and precancerous conditions in other locations, such as the female genital tract\textsuperscript{17,45–47} or liver,\textsuperscript{48} and benign conditions, like epithelial expansion in ovarian endometrial cysts\textsuperscript{49} or focal nodular hyperplasias of the liver.\textsuperscript{50}

DNA-ploidy results also confirmed the differences between ACNHs and ACAs, supporting the neoplastic nature of monoclonal ACAs. Nondiploid DNA contents were found in 40% (10/25) of ACAs and in only 5% (1/20) of ACNHs. Those cases preferentially revealed hyperdiploid G\textsubscript{0}/G\textsubscript{1} cells and monoclonal proliferations in 86% of informative cases. DNA content analysis has been reported to be useless as a diagnostic tool to differentiate benign from malignant conditions.\textsuperscript{51} Although hyperdiploid DNA content was also found in one ACNH, the close association between DNA aneuploidy and monoclonal proliferation supports a neoplastic nature, especially when they are associated.

In conclusion, both ACNHs and ACAs are kinetically defined by low-apoptotic cell growths. However, a distinctive correlation between proliferation and apoptosis, direct for ACNHs and inverse for ACAs, helps explain clone selection. That inverse correlation of kinetic parameters would provide the best selective mechanism resulting in dominant clone expansion (monoclonal proliferation) in ACAs whereas direct correlation gives a less selective mechanism, allowing balanced expansion of clones (polyclonal proliferations) in ACNHs.

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Clonal patterns in phaeochromocytomas and MEN-2A adrenal medullary hyperplasias: histological and kinetic correlates

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Abstract

The relationship among histological features, cell kinetics, and clonality has not been studied in adrenal medullary hyperplasias (AMHs) and phaeochromocytomas (PCCs). Thirty-four PCCs (23 sporadic and 11 MEN-2A (multiple endocrine neoplasia type 2A)-related tumours, the latter associated with AMH) from females were included in this study. Representative samples were histologically evaluated and microdissected to extract DNA and evaluate the methylation pattern of the androgen receptor alleles. At least two tissue samples (from the peripheral and internal zones in each tumour) were analysed with appropriate tissue controls run in every case. The same areas were selected for MIB-1 staining and in situ end labelling (ISEL). Malignant PCCs were defined by histologically confirmed distant metastases. All monoclonal AMH nodules from the same patient showed the same X-chromosome inactivated. Six sporadic PCCs revealed liver metastases (malignant PCC) and eight additional sporadic PCCs showed periadrenal infiltration (locally invasive PCC). All informative PCCs were monoclonal, except for five locally invasive PCCs and one benign PCC that revealed polyclonal patterns. Those cases also showed a fibroblastic stromal reaction with prominent blood vessels, focal smooth muscle differentiation, and significantly higher MIB-1 (126.8 ± 29.9) and ISEL (50.9 ± 12.8) indices. Concordant X-chromosome inactivation in nodules from a given patient suggests that MEN-2A AMH is a multifocal monoclonal condition. A subgroup of PCCs characterized by balanced methylation of androgen receptor alleles, high cellular turnover, and stromal proliferation also shows locally invasive features. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: phaeochromocytomas; adrenal medullary hyperplasias; MEN-2A; X-chromosome inactivation; proliferation; apoptosis; stromal reaction

Introduction

Phaeochromocytomas (PCCs), either sporadic or associated with multiple endocrine neoplasia type 2 (MEN-2), are arbitrarily distinguished from adrenal medullary hyperplasias (AMHs) [1,2], especially for large nodules in a multinodular background [3]; some reports have proposed nodule size (>1 cm) as the differentiating criterion [2,4]. Our knowledge of AMH comes from inherited conditions, especially MEN-2, where morphometry has been shown to be useful in distinguishing AMH from normal adrenal medulla [3]. The current diagnosis of malignant PCC requires the demonstration of metastases, defined by tumour growths in sites where chromaffin tissue is not present, such as lymph nodes, liver, and bone [2]. Locally invasive PCCs are characterized by variable invasion of periaudrenal tissue and no evidence of distant metastases [5–7], but the association of local invasion with lymph node metastases and long-term outcome remain unknown [2]. Neoplasms are defined as clonal proliferations, in contrast to the heterogeneous composition of most normal tissues [8,9]; hence a monoclonal cell population which has arisen from heterogeneous normal tissues strongly suggests a neoplastic nature [7,10]. Different and variable success has been reported for several markers, but clonality still remains the hallmark of neoplasia. Clonal selection should also be the expression of kinetic advantage (high proliferation or abnormally low apoptosis) [7,10–12] resulting in dominant growths [7,8]. The relationship of clonality, cell kinetics (proliferation and apoptosis), and histological features has not been studied in AMH and PCC.

This study investigates the clonal pattern of sporadic and MEN-2A-associated AMH and PCC, based on the analysis of the methylation pattern of androgen receptor alleles (ARAs) using microdissected samples,
and its correlation with kinetic and histological features.

Materials and methods

Case selection

Thirty-four PCCs from females (23 sporadic and 11 MEN-2A-related) were studied. Six sporadic PCCs were malignant (histologically confirmed liver metastases and elevated post-operative levels of catecholamines) and eight sporadic PCCs were locally invasive (periadrenal infiltration). The post-operative catecholamine levels were normal in both locally invasive and benign PCCs. Familial PCCs were associated with AMH, selected from 177 members (five generations) of known MEN-2A kindred and classified as benign. These patients were screened for C-cell hyperplasia – medullary thyroid carcinoma and demonstrated to carry a germline point mutation on codon 634 of the RET proto-oncogene by PCR-restriction fragment length polymorphism and sequencing [13]. AMH was defined according to standard criteria (expansion of the medullary compartment into areas of the gland where it is not normally present) [3], considering nodules larger than 1 cm as PCC [2,4].

All surgical specimens were serially sectioned, sampled according to standard protocols (1 block/cm of tumour-nodule), and had appropriate archival material available. The same areas in consecutive sections were used for each study and their cellular composition was confirmed in adjacent haematoxylin and eosin-stained sections.

Clonality analysis

DNA was extracted from two 20-μm unstained paraffin sections that included at least 100 cells per sample (about 0.4 mm²), using a modified phenol-chloroform protocol [14]. Microdissected samples were systematically taken from the peripheral and internal zones of every tumour. The small size of most nodules precluded reliable topographic sampling; one sample was taken from the peripheral area of each nodule. Appropriate controls were included for each test (adrenal medulla, adrenal cortex, and periadrenal soft tissue). Only well-defined nodules from patients with a polyclonal pattern in the intervening adrenal medulla were included, to guarantee appropriate controls.

Half of each sample underwent Hha-I digestion (0.8 unit/μl; New England Biolabs, Beverly, MA, USA) and the other half was used as an undigested control. Both samples were equally processed, but excluding Hha-I in the undigested ones [10,15–17]. A mimicker (0.3 μg of double-stranded and Xho I-linearized φX174-R11 phage) (Gibco-BRL, Gaithersburg, MD, USA) was included to test digestion completion after gel electrophoresis; incompletely digested samples were repurified and redigested with a higher Hha-I concentration. Hha-I was inactivated by phenol-chloroform extrac-

tion [14] and DNA was precipitated with ice-cold absolute ethanol in the presence of 0.3 m sodium acetate, pH 5.2 and resuspended in 10 μl of 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, and 100 μg/ml BSA. The first exon CAG repeat of the androgen receptor gene was amplified, according to the conditions shown in Table 1, in a Perkin-Elmer thermal cycler model 480 (Perkin-Elmer, Norwalk, CT, USA), using both digested and undigested DNA templates [16,17].

The whole PCR volume (10 μl) was electrophoresed into 8% non-denaturing polyacrylamide gels (0.75 mm) and run at 5 V/cm until the xylene cyanol band was within the bottom gel inch. The gels were fixed with 7% acetic acid (5 min), dried in vacuo (40 min, 80°C), and put inside a developing cassette containing one intensifying screen and preflashed films (Kodak XAR) facing the intensifying screen (16–48 h, −70°C). The autoradiograms were developed using a Kodak-Omat 100 automated processor (Kodak Co., Rochester, NY, USA).

Interpretation and inclusion criteria were as previously reported [10,18]. Only informative cases (two different alleles in undigested and digested controls) were included in the final analysis [10,16,17]. Allelic imbalance was densitometrically evaluated (EC model 910 optical densitometer, EC Apparatus Corporation, St Petersburg, FL, USA), using both digested and undigested DNA templates [16,17].

In situ end labelling (ISEL) of fragmented DNA

Extensive DNA fragmentation associated with apoptosis was detected by ISEL as previously reported [12,19]. Sections were routinely deparaffinized, hydrated, and incubated with 2 × SSC buffer (80°C, 20 min). After pronase digestion (500 μg/ml, 25 min, room temperature), the sections were incubated with the Klenow fragment of Escherichia coli DNA polymerase I under appropriate conditions (20 units/ml, 100 μM of each dNTP with a proportion of 11-digoxigenin-dUTP/dTTP of 0.35/0.65, 2 h at 37°C). The digoxigenin-labelled DNA fragments were detected using anti-digoxigenin polyclonal antibody labelled with alkaline phosphatase (1/100 dilution; Boehringer-Mannheim, Germany). The reaction was developed with nitroblue-tetrazolium and X-phosphate under microscopic control [12], and the sections were counterstained with diluted haematoxylin (25%). Appropriate controls were run, including positive (reactive lymph node), negative (omitting DNA polymerase I), and enzymatic (DNase I digestion before end labelling). The enzymatic controls allowed a reliable positivity threshold to be established in each sample.
Immunohistochemical expression of MIB-1, S-100 protein, smooth muscle actin, and desmin

The sections were mounted on positively charged microscope slides (Superfrost Plus, Fisher Scientific, NJ, USA), baked at 60°C for 2 h, and routinely dewaxed and rehydrated. The endogenous peroxidase activity was quenched (0.5% H₂O₂ in methanol, 10 min) and the antigens were retrieved (20 min in 10 mM citrate buffer, pH 6.0, using microwave treatment at 600 W). After treatment with polyclonal horse serum (20 min, 1/100 dilution) (Dako, Denmark), the sections were incubated with monoclonal primary antibodies (overnight, 4°C) at 1 μg/ml for MIB-1 (Oncogene Science, Cambridge, MA, USA), S-100 protein, smooth muscle actin, and desmin (Dako, Denmark). The reaction was developed using biotinylated anti-mouse antibody (30 min, 1/200 dilution) (Dako, Denmark), peroxidase-labelled avidin–biotin complex (60 min, 1/100 dilution) (Dako, Denmark), and 3,3’-diaminobenzidine tetrahydrochloride with 0.3% H₂O₂ as chromogen (Sigma Co., St. Louis, MO, USA). The sections were counterstained with haematoxylin. Both positive and negative (omitting the primary antibody) controls were simultaneously run.

Quantification of positive nuclei

At least 50 high power fields (HPF), or the complete lesion if smaller (50 HPF = 7.6 mm²), were screened in each pathological group, beginning in the most cellular area. Both the number of positive nuclei per HPF and the number of proliferating cells intercepted by the microscope field diameter were registered. The last score and the formula $N = (πr²/4)$ ($N$ is the number of estimated cells per HPF and $n$ is the number of cells intercepted by the microscope field diameter) [20,21] were used to estimate the number of proliferating cells per HPF. Both the average and the standard deviation (SD) were calculated in each patient and pathological condition and expressed per 1000 proliferating cells. Only nuclei with staining features similar to those of their corresponding positive control were counted for a given parameter (MIB-1, ISEL).

Statistical analysis

The average and SD of every quantitative variable were compared in each pathological condition (adrenal medullary hyperplasia, benign PCC, locally invasive PCC, and malignant PCC) and per tumour/nodule compartment by two-tailed Student’s $t$-test or analysis of variance (ANOVA) for non-parametric distribution, and considered statistically significant if $p < 0.05$. 

Results

We found 34 AMH nodules in 11 MEN-2A patients (three patients contributing two nodules, four cases with three nodules, and four adrenals with four nodules). Four nodules from two patients (two nodules each) were considered non-informative and excluded from further analysis. Twenty-seven nodules revealed the same X-chromosome inactivated in nodules from the same patient (Figure 1). The remaining three nodules (from two patients) showed a polyclonal pattern. As no reliable differences accurately distinguished true polyclonal lesions from clonal proliferations of two divergent cell populations, these cases were also excluded.

Concordant methylation patterns of ARAs were found in nine informative patients: one contributing two nodules, four providing three nodules, and four giving four nodules. Cells from informative patients inactivate either the smaller or the larger allele, resulting in a 50% probability of finding the same allele methylated in cell-to-cell comparison. Assuming an equal and independent probability of ARA methylation at the cellular level, each tissue from an informative female can be revealed to be polyclonal, monoclonal with preferential methylation of the larger allele, or monoclonal with the smaller allele predominating [22]. Equal a priori probability for each pattern should be expected ($p = 1/3$) and tissue ARA patterns from informative patients would be concordant with a probability of 2(1/3)⁹, where 2 is the number of alleles and $n$ is the number of lesions compared. Therefore, identical methylation patterns of ARAs in these nine patients would be randomly found with a probability of $2 ((1/3)^3)(1/3)^4 ((1/3)^3)^3 = 2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 (1/3)^2 = 2 (1/3)^{30} = 9.71 \times 10^{-15}$.

Concordant methylation patterns of ARAs were observed in peripheral and internal samples of each PCC. Of 29 informative patients with PCC, 23 PCCs revealed a monoclonal pattern (17 benign, including eight sporadic and nine in MEN-2A patients, and six malignant) and six sporadic PCCs (five locally invasive

Table 1. Primer sequences and PCR cycling conditions for the amplification of polymorphic DNA regions

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequences</th>
<th>Tandem repeat/PCR product</th>
</tr>
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<tbody>
<tr>
<td>AR-a</td>
<td>5’-CCG AGG AGC TTT CCA GAA TC -3’</td>
<td>CAG repeat/215–300 bp</td>
</tr>
<tr>
<td>AR-b</td>
<td>5’-TAC GAT GGG CTT GGG GAG AA -3’</td>
<td></td>
</tr>
</tbody>
</table>
and one benign) showed a polyclonal pattern (Figure 2). Histology revealed a distinctive stromal reaction in three AMH nodules and six PCCs (all polyclonal), comprising prominent spindle cell proliferation with focal cytoplasmic expression of smooth muscle actin and desmin and prominent blood vessels (Figure 3). Stromal contamination was excluded by repeated careful microdissections that confirmed the same polyclonal pattern. No differences were detected whether or not the samples were boiled during extraction.

A progressive increase in both MIB-1 and ISEL indices was revealed in the sequence AMH – benign PCC – malignant PCC, but with no statistically significant differences (Table 2). The only significant differences were observed in locally invasive PCC, which showed the highest indices \( p < 0.001 \) (Table 2 and Figures 3 and 4). No topographic differences were detected for any marker or pathological condition (Table 2).

**Discussion**

Different conclusions emerged for AMH and PCC. AMH nodules in MEN-2A have been proven to be mainly monoclonal and have revealed the same X-chromosome inactivated in a given patient; this suggests that clonal expansions target precursors before they spread in the adrenal medulla and that nodular AMH is a clonal and multifocal lesion. In sporadic PCC, a distinctive histological and kinetic profile correlates with a polyclonal pattern and periadrenal infiltration.

Monoclonal AMH nodules (27/30 informative nodules, 90.0%) showed the same ARA preferentially methylated in a given patient, suggesting that a common progenitor contributed to such lesions and supporting multifocal rather than multicentric growth, as proposed for other lesions [23–25]. The internodular adrenal medulla had to be polyclonal to consider the case informative, thus precluding any conclusion for diffuse AMH. The presence of monoclonal AMH nodules which have developed in polyclonal diffuse AMH supports their neoplastic nature [8,9] and a multistep tumourigenesis in the adrenal medulla of MEN-2A patients [3,4,26]. Their concordant monoclonal pattern in a given patient supports early clonal expansion of precursor cells between the random X-chromosome inactivation and their spreading in the adrenal medullary anlage [7,10], resulting in PCC when other genes are targeted and genetic alterations accumulate. Supportive evidence showed at least one microsatellite abnormality of tumour suppressor genes in 75% of MEN-2A PCCs (manuscript submitted) and the same X-chromosome preferentially inactivated in both thyroid lobes in monoclonal C-cell hyperplasias from 8/9 informative patients from these kindreds (89%) (manuscript submitted). However, two other reasons for monoclonal patterns must be excluded. Firstly, contiguous cellular regions of the same lineage (‘patch size’ concept) and embryological factors determine the clonal pattern in polymorphic tissues. Any kinetic advantage of cell groups sharing the same inactivated X-chromosome would determine their preferential growth and mosaic patch size (non-random skewness of X-chromosome inactivation) in the early stages [7,8,10,11]. Our controls and sampling technique \(( \geq 100 \) cells\) exclude non-random skewness or patch size variability as causes [7,10]. In addition, patch size mosaicism could not explain AMH nodules with concordant X-chromosome inactivation. Secondly, PCR bias against the larger ARA could contribute to preferential amplification of the smaller ARA. Our DNA extraction protocol [14,27] included a long protein digestion and demonstrated an average DNA size of about 1 kb (data not shown), excluding DNA degradation as the cause. Our PCR design [10,17,28,29] also included long denaturation – extension in the first three cycles and 7-deaza-dGTP in the amplification mixture to exclude PCR bias (Table 1), as confirmed in the cases showing the larger ARA methylated (Figure 2).

The first clonality study in MEN-2A PCC reported only one glucose-6-phosphate dehydrogenase isoenzyme in tumour tissue from patients heterozygous for
that marker [30,31]. The authors suggested initial mutations producing multiple clones of defective cells as a preneoplastic condition, which would progressively evolve into monoclonal growths by clone selection [30,31]. Although the histopathological features have not been characterized, the initial stage in our study would be the polyclonal diffuse AMH that results in monoclonal nodular AMH by early clonal expansions. The migration of neural crest cells into the adrenal medulla proceeds in finger-like projections around the blood vessel that penetrate the cortex from the caudal aspect [32] and help to explain the multinodular pattern if early clonal expansion affects the precursor cells. Our results also question the diagnostic utility of nodule size to distinguish AMH from PCC [2]. No reliable histological criterion distinguishes large nodules in a multinodular AMH from small PCC, and morphometry only distinguishes AMH from normal adrenal medulla [3]. Tumour-nodule size is a time-dependent parameter that revealed a monoclonal pattern even in small nodules, an unsurprising finding in processes with early neoplastic transformation, such as most inherited tumour syndromes [33,34], including MEN-2.

Monoclonal PCC (23/29, 79.3%) revealed the same X-chromosome inactivated in the peripheral and internal compartments. All MEN-2A PCCs revealed this feature and multinodular AMH, in agreement with the high prevalence of monoclonal patterns found in these AMH nodules (27/30, 90%). A polyclonal pattern was revealed in sporadic locally invasive PCCs (6/29, 21.7%), which could be explained under the three following circumstances. Firstly, X-chromosome inactivation was tested using methylation-sensitive restriction endonucleases. Both incomplete endonuclease digestion [7] and aberrant hypermethylation (due to tumour progression or abnormal imprinting) [10] would result in false polyclonal patterns in monoclonal tissues. The completion of endonuclease digestion was tested using a viral mimicker and both the long denaturation (16 h) and the activity of Hha-I on single-stranded DNA would ensure complete digestion of the denatured DNA related to embedding and extraction. Repeated polyclonal patterns were obtained using non-boiled templates. We are currently testing methylation in these tumours. Secondly, any significant contamination with normal cells could be excluded by a consistent polyclonal pattern after careful and repeated microdissection. Contamination with normal cells would determine a polyclonal gel pattern in monoclonal tissues [7,10]. Other systems such as laser-capture microdissection can decrease contamination, but the results reported for samples of around 100 cells are identical using manual or laser-capture microdis-

**Figure 2.** Histology and clonality in phaeochromocytomas (PCCs). Concordant methylation patterns of androgen receptor alleles were found in peripheral and internal samples from the same tumour. Polyclonal cases (right-hand side of the panel) revealed stromal proliferation with balanced methylation of androgen receptor alleles (Haematoxylin and eosin, × 300 left and × 150 right). D = digested sample.
section [35]. In the present series, the stromal reaction was revealed as polyclonal and excluded in careful microdissections, but PCC still showed a polyclonal pattern. These non-epithelial components have been proposed as a key element of epithelial growth by either secretion of stimulatory factors or lack of an inhibitory factor [36]. Thirdly, clonal proliferation of cells showing different X-chromosomes inactivated (as expression of an unfinished process of cell selection) also results in polyclonal patterns, but requires additional markers such as loss of heterozygosity of tumour suppressor genes [7] to prove it (manuscript in preparation). These polyclonal patterns would represent true polyclonal proliferations only if precursor clonal expansions occurred after the random X-chromosome inactivation [7,10].

The second remarkable aspect of PCC with a polyclonal pattern was the high cellular turnover demonstrated by high MIB-1 and ISEL indices. We have observed endocrine tumours with regressive changes (stromal proliferation, prominent blood vessels, and increased apoptosis index) (Figure 5) [11,29], but with a positive proliferation–apoptosis ratio and slow tumour growth. They also showed variable periadrenal invasion and stromal overgrowth with smooth muscle differentiation, but no evidence of distant metastases. Stromal and vascular proliferations in tumours have been described as a reaction to

Table 2. Quantification of nuclear MIB-1 expression and in situ end labeling in adrenal medullary hyperplasias and pheochromocytomas by topographic compartment

<table>
<thead>
<tr>
<th></th>
<th>MIB-1 index</th>
<th>ISEL index</th>
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<tr>
<td></td>
<td>Peripheral</td>
<td>Internal</td>
</tr>
<tr>
<td>MEN-2A AMH</td>
<td>15.3 ± 3.9</td>
<td>16.7 ± 3.6</td>
</tr>
<tr>
<td>PCC</td>
<td>28.3 ± 4.3</td>
<td>31.1 ± 5.6</td>
</tr>
<tr>
<td>Benign</td>
<td>30.2 ± 4.4</td>
<td>33.7 ± 5.3</td>
</tr>
<tr>
<td>Locally invasive</td>
<td>126.8 ± 29.9</td>
<td>132.5 ± 33.2</td>
</tr>
<tr>
<td>Malignant</td>
<td>70.1 ± 20.6</td>
<td>74.0 ± 19.9</td>
</tr>
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invasion or as an expression of regression. Interstitial fibrin deposition from leaky tumour vessels provides a provisional stroma that serves to regulate the influx of inflammatory cells and facilitates the inward migration of new blood vessels and fibroblasts, the mature tumour stroma [37]. That process has demonstrated a pivotal role in promoting tumour growth [38]. Those features present in locally invasive PCC would explain the high proliferation rates and the presence of tetraploid cells [39] secondary to non-disjunctional mitoses. This cellular process would end in lethal genetic changes and an increased apoptotic rate (Figure 5). We can speculate that the expansive process would contribute to the periadrenal invasion in a non-metastasizing neoplasm, but it needs additional studies, especially focused on tumour cell adhesion and its association with lymph node metastases and patient outcome.

In conclusion, nodular AMH is essentially a multifocal monoclonal proliferation that shows the same X-chromosome inactivated in AMH nodules from a given patient, suggesting an early clonal expansion of adrenal medullary precursors. A subgroup of PCCs with a polyclonal pattern, high cellular turnover, and stromal proliferation also shows locally invasive features. This group needs additional studies to define its biological meaning.

References


Clonality Studies in the Analysis of Adrenal Medullary Proliferations: Application Principles and Limitations

Salvador J. Diaz-Cano, MD, PhD

Abstract

Clonality remains as the hallmark of neoplasms. A dual genetic approach using markers nonrelated (e.g., X-chromosome inactivation assays) and related to the malignant transformation (such as loss of heterozygosity analyses of tumor-suppressor genes) would provide useful clonality information from early and advanced tumor stages, respectively. Tumor progression and clonal selection would result in genetic instability and heterogeneous expression of those molecular markers related to the malignant pathway. Therefore, only the coexistence of multiple genetic abnormalities would support the clonal nature as an expression of convergent cell selection. Considering those facts, the currently available evidence on tumorigenesis and clonality in the adrenal medulla can be summarized as follows:

1. Multistep tumorigenesis defines the evolution of pheochromocytomas, as evidenced by the presence of several genetic alterations.
2. Both the significant association of nonrandom genetic alterations (specially 1p and 22q interstitial deletions) and the topographic accumulation of genetic deletions at the peripheral tumor compartment support a convergent clone selection for these neoplasms.
3. Although many genetic loci show nonrandom abnormalities, the most frequently involved locates on chromosome 1p regardless of genetic tumor background (sporadic or inherited predisposition).
4. Most pheochromocytomas should begin as monoclonal proliferations that do not always correlate with histopathologic features, particularly in inherited tumor syndromes.
5. Early histopathologic stages, described as adrenal medullary hyperplasias, are defined by hyperproliferative features in animal models and monoclonal patterns in the adrenal nodules from patients with MEN-2a.

Key Words: Pheochromocytoma; adrenal medullary hyperplasia; clonality; X-chromosome inactivation; loss of heterozygosity; clone selection; multistep carcinogenesis.

Introduction

Although many molecular features have been described in order to characterize neoplasms better, its definition is still descriptive. All these features try to distinguish neoplasms from nontumoral conditions and malignant from benign neoplasms as well. Malignant tumors (specially carcinomas) evolve through continuous and additive changes, both at genetic and phenotypic levels, ending in the complete malignant phenotype. The most widely accepted theory proposes a clonal origin for them, in contrast to the heterogeneous composition in most normal tissues [1,2]. Therefore, monoclonal cell populations
arisen from heterogeneous normal tissues strongly suggest a neoplastic nature. Different and variable success has been reported for several markers, but clonality still remains as the hallmark of neoplasms. Before any specific reference to clonality in pheochromocytomas and related lesions of the adrenal medulla, some general biologic considerations would help us to understand the strength and weakness of molecular techniques in the analysis of clonality. Those considerations will focus on the relationship among cellular kinetics, neoplastic transformation, and clonality. A brief review of the morphologic criteria of pheochromocytomas (PCC) and adrenal medullary hyperplasia will follow, and finally, the current information on clonality in the analysis of adrenal medullary proliferations will be covered.

General Considerations on Malignant Transformation and Clonality

The term clonality has been broadly used, and it is related to the presence of cellular clones in tissues, although that terminology can be applied at different organizational levels. Those clones represent the groups of genetically identical copies of a single progenitor. The strict application of that definition would require the sequence of all DNA in order to exclude any polymorphism, but that approach is not viable. Therefore, different markers have been proposed to obtain such information using more or less complicated and simple techniques. In any case, the presence of groups of genetically identical cells must be related to proliferative advantages of those cells over the remaining. For that reason, a close relationship can be drawn between clonality and kinetics features. The expression of those features is variable from the conventional histologic level to the molecular one.

The current definition of neoplasm is descriptive and closely related to clonality. Neoplasms are defined as abnormal and self-maintained proliferations, resulting in the presence of new tissues. Those new tissues should come from a group of cells sharing a particular and common advantage. Therefore, a clear relationship between both concepts (neoplasm and clone) is present. Its application to everyday practice of pathology would also contribute to explaining the development of diagnostic criteria of neoplasm in many areas of surgical pathology. Just as an example, the diagnosis of malignant lymphomas, especially at the extranodal level, requires the presence of a monotonous proliferation of lymphoid cells that substitutes the normal tissue architecture and results in a mass. Those criteria represent, at the histologic level, the morphologic evidence of an expansive growth of neoplastic cells. Sometimes, those cellular expansions share specific phenotypes (cytoplasmic or surface markers, and so forth), which allow their identification. Such an approach would require quantification of its expression in order to distinguish the abnormal proliferation from its normal counterpart, e.g., light-chain restriction of immunoglobulin in lymphoid neoplasms or the flow cytometric analysis of surface lymphoid markers in hematologic malignancies.

On the other hand, the neoplastic transformation is a multistep process, resulting in a complex mixture of tissues with heterogeneous genetic background [1]. The results of every molecular test will depend on the timing between the evaluated biologic feature and the specific marker (Fig. 1). Simultaneously, the sensitivity and specificity of each molecular marker used to test any biologic feature, such as clonality,
Fig. 1. Timing between the clonal signal stimulus and the X-chromosome inactivation (XCI). The interpretation of any test result (in this case XCI) will depend on the temporal relationship between the marker used (methylation of inactive alleles on X chromosome) and the biologic feature evaluated (in this case clonality). Normally XCI takes place early before any clonal signal (labelled + in the figure), ending in monoclonal patterns (A). Those situations where the clonal signal precedes the XCI would eventually result in polyclonal patterns, even for monoclonal proliferations ("pseudo-polyclonal", B).
should be checked with samples of different and known genetic constitution. In addition, those molecular markers of clonality should be evaluated in relation to two main features, cellular kinetics and the neoplastic pathway. Any marker related to the malignant pathway will provide positive information only if that target participates in the molecular pathway of a given tumor. The current evidence suggests that malignant tumors result from the accumulation of multiple genetic events rather than from any specific sequence of genetic changes [3–5]. Some mutations are more important than others, because they facilitate additional phenotypic or genotypic changes. This reason explains certain trends in the order of mutation production, although significant variation for them is the role [6]. The lack of such a sequence and the unpredictability of genetic changes in malignancies preclude the extensive clinical use of those genetic markers with diagnostic and prognostic purposes. Finally, the timing between the clonal expansion and the presence and expression of malignant features is unknown. All these limitations should be considered to achieve reliable and useful information from any marker.

The final expression of any neoplasm depends on the progressive and convergent selection of cell populations, but several factors should be taken into consideration. On one hand, that selection will determine tumor progression and cellular heterogeneity. On the other hand, cellular selection is the expression of cell kinetics [7]. Considered from the genetic point of view, neoplasms can be characterized by multiple genetic aberrations, from gross to point mutations. However, those genetic aberrations should be “fixed” on the transformed cell before ending in a fully established malignancy. Any nonlethal genetic aberration will be “cell-fixed” if both its transmission to descendant cells is warranted and it is able to bypass the cell-repairing systems. Otherwise, any genetic alteration leading to cellular aging, differentiation, or activation of the apoptotic pathway will be nontumor-productive (Fig. 2). This process determines a complex network where a potential genetic marker will be useful when associated with kinetic advantages responsible for cellular outgrowths.

It is out of the scope of this article to provide detailed descriptions of those markers used for testing both clonality and cell kinetic [7–9]. Essentially, two types of molecular markers provide reliable results on clonality, classified according to its chromosomal location in X-linked and non-X-linked assays. Those markers independent of the malignant transformation pathway provide more general information, especially from the early neoplastic stages. Among them, the assays based on the random inactivation of one X-chromosome in females are the most extensively used to test clonality [9]. These tests target the differential expression of alleles of X-chromosome-linked genes (mRNA or protein analyses) or the differential DNA methylation on active (nonmethylated) and inactive (methylated) alleles. Currently, the most productive marker of this sort is the human androgen receptor gene (HUMARA) owing to its high percentage of heterozygosity in the general population (around 90%) related to the presence of a highly polymorphic trinucleotide repeat region. That polymorphic region also shows a predictable methylation pattern on sequences recognized by methylation-sensitive restriction endonucleases [10], allowing allele differentiation. In fact, the information on clonality provided by both X-linked and non-X-linked assays is complementary and nonmutually exclusive, because the results from X-linked
assays are based on genetic features usually nonrelated to the malignant transformation. The inactivation of one X-chromosome in females is an early event during embryogenesis, described in embryos of 16–30 cells [11]. Two main consequences can be drawn, with both biologic and technical implications. First, some tissues would show the same allele inactivated and a monoclonal pattern, avoiding any positive conclusion from a tumor arisen on it (noninformative case) [2,7]. This pattern is variable from patient to patient and even from tissue to tissue in the same patient. For that reason, the so-called Lyonization ratio, reliable and appropriate results for any tumor or precancerous tissue samples must require embryologically-related controls [12,13]. Second, most of somatic genetic alterations described in neoplasms will take place on cells showing one X-chromosome inactivated. Those genetic events associated with kinetic advantages and cellular overgrowths will probably determine a monoclonal methylation pattern of the X-chromosome if they trigger one cell or a small group of neighboring cells (Fig. 1).

Those tests based on other somatic markers potentially involved in the malignant pathway give information on the transformation mechanism. Any nonrandom genetic alteration (X-linked or not) present in most of the tumor cells will provide useful information on clonal expa-
sion, even those as small as point mutations. Those point mutations, regardless of their location on exons or introns (the latter, also referred to as single nucleotide polymorphism, has no functional expression at mRNA and protein levels), would be related to kinetic advantages and, therefore, have been proven useful to test clonal expansions in tumors. However, only those tumors expressing that particular marker will be suitable for that analysis. Additionally, the ratio of positive results will be necessarily related with both time-independent and time-dependent features of each tumor, e.g., histologic type, location, grade, or stage. Those variables can be “confusing factors” if they are not adequately evaluated. Owing to the absence of an established timing for a particular genetic event in the transformation pathway, variably long gaps of test negativity would be demonstrated for a particular molecular marker in a given tumor (Fig. 3). That would cover the time between tumor initiation and assay conversion, giving inconclusive results. There will not be any gap time for such conversion if the tested molecular marker represents the first mutation event in the natural history of a given neoplasm. In that case, the test results would be identical to those provided by markers unrelated to the malignant transformation pathway, e.g., X-chromosome-inactivation assays. In conclusion, only multifactorial approaches considering different markers simultaneously would appropriately provide clonality information in tumor conditions.

**Pheochromocytomas and Adrenal Medullary Hyperplasias: Basic Pathology**

In general, the morphologic features of endocrine tumors barely correlate with biologic behavior. Many clinicopathologic studies make the point that cytologic features, such as nuclear pleomorphism, hyperchromatism, or increased nuclear/cytoplasmic ratios, are not necessarily related with aggressive outcome. Sometimes, only the presence of metastatic tumor growths enable us to establish a malignant diagnosis [14]. Therefore, in addition, histopathological studies are required to improve both diagnosis and prognosis of these neoplasms. Other approaches trying to detect the tumor at early steps (precancerous conditions or incipient neoplasms) go in a parallel direction to improve patient prognosis. Pheochromocytomas (PCC) fulfill all these general rules.

The histopathologic differentiation of hyperplastic and neoplastic conditions in the adrenal medulla is mainly arbitrary [14,15]. The first differentiating criterion is the increase in the adrenal weight, as an expression of the increased number of chromaffin cells, with expansion of the medullary compartment into areas of the gland where it is not normally present. Morphologically, hyperplastic conditions have been classified according to their growth pattern as diffuse or nodular [16]. Additionally, no specific and consistent criteria will distinguish big nodules within a multinodular background (hyperplastic condition) from small PCC (neoplastic condition). Some reports propose the nodule size as the main differentiating criterion and 1 cm as the threshold [17]. That size has been suggested as the criterion because it is the detection limit of most image diagnostic systems and represents the smallest size clinically detectable. That tumor size was also selected because the smallest PCC in the first series from the Armed Forces Institute of Pathology had 1 cm diameter [15]. No biologic reasons are provided for that distinction. On the other hand, the knowledge available for adrenal medullary hyperplasia comes from inherited condi-
PCCs have been described as sporadic neoplasms or associated to other tumors as part of inherited cancer syndromes, mainly multiple endocrine neoplasia type 2 (MEN-2), neurofibromatosis, or von Hippel-Lindau disease [18]. Clinically and histopathologically, the situation is differ-

Fig. 3. Natural history of neoplasms based on clone selection and tumor progression. The natural history of neoplasms is a linear temporal expression of the convergent and endless selection process of cell clones in tumors. The clone selection results from the interaction between genetic changes (mutational events) and individual susceptibility. Progressive and cooperative changes should accumulate in cells before they are expressing a fully neoplastic phenotype. The point of irreversibility in that sequence also separates the chronologic evolution from preneoplastic into neoplastic conditions. On that background, the clonality markers will give useful information to depict the neoplastic natural history. Any particular marker will give information concerning clonality if it is involved in the malignant transformation process. Likewise, it will be concordant with XCI assays only if it represents the first mutational genetic event. All other situations will determine negative results for the marker even for already clonal proliferations (negative test gap).

Cytologic and architectural features have showed no significant and reliable differences useful for that task [15].
The absence of either a known genetic background or a reliable screening method precludes the systematic detection of early sporadic tumors. Therefore, most of those sporadic tumors are detected in more advanced stages, although only about 10% shows malignant behavior. Additionally, no preneoplastic changes are usually associated with them. The opposite situation characterizes the inherited PCC: they are usually detected earlier owing to genetic and clinical screening, and medullary hyperplasia, either diffuse or nodular, is also present.

Clinicopathologic studies mainly define benign and malignant PCCs. Currently, a bona fide diagnosis of malignant PCC requires the demonstration of metastases, defined by the presence of tumor overgrowths in sites where chromaffin tissue is not normally present. Those organs include lymph nodes, liver, and bone. The presence of nonneoplastic chromaffin tissue in other organs (as a physiologic finding or as a part of hyperplastic conditions) avoids the establishment of malignant diagnoses based merely on the histopathologic evidence of such tissue. The demonstration of truly metastatic outgrowths would require additional studies in order to prove clonal identity in both primary and metastatic tumor. Relatively clear patterns are usually shown at both ends of the pathologic spectrum (benign and malignant tumors). A slightly different situation would be expected in borderline conditions, such as locally invasive tumors. Those tumors are characterized by variable invasion of periadrenal soft tissue, with no evidence of distant metastatic growths. Its association with lymph node metastases needs to be defined and the long-term patient outcome is unknown.

Clonality Analyses of Adrenal Medullary Proliferations

Genetic analyses of different molecular markers, especially those of polymorphic DNA regions, have provided valuable information on the pathogenesis of endocrine neoplasms, both sporadic and familial. Those polymorphic DNA areas allow reliable allele identification, and, therefore, DNA loss would be expressed as reduction to hemizygosity. According to Knudson's hypothesis, DNA loss would explain neoplastic development and progression if tumor-suppressor genes are involved. A relatively common pattern for most PCCs came from those studies, showing genetic deletions (demonstrated by loss-of-heterozygosity [LOH] analyses) at several loci, particularly at 1p, 3p, 11p, 17p, 17q, and 22q. Those interstitial DNA deletions mainly involve both known tumor suppressor genes, such as von Hippel-Lindau gene locus (3p25), neurofibromatosis 1 locus (17p), or p53 locus (17p), and unknown tumor-suppressor genes at 1p (at 34–36, distal to D1S73 and proximal to D1S63), 11p, 11q, and 22q (distal to D22S24 and proximal to D22S1). Additionally, activating point mutations of genes involved in other endocrine tumors have been demonstrated in both sporadic and familial PCCs, including ret proto-oncogene and the G-protein gene encoding for Gsα (gsp).

Evidences of Clonality Based on X-Chromosome Inactivation Analysis

Preliminary results revealed monoclonal methylation pattern in the alleles of the androgen receptor gene (HUMARA assay of clonality) in 87% of informative cases from a series of sporadic PCCs. After microdissection, at least two different
samples were analyzed in each neoplasm, from the peripheral and the internal tumor areas, respectively. Both tumor areas provided concordant methylation patterns, consistent with a monoclonal origin for this series of cases. The remaining 13% of informative PCCs showed balanced methylation of both HUMARA alleles, pointing to a polyclonal tumor growth. The latter group of cases corresponded to locally invasive PCCs (as defined by periarudral soft tissue invasion) with high cell turnover rate (both MIB-1 labeling index and in situ end-labeling index received the highest scores for this series) [21]. Histologically, stromal overgrowth with a prominent smooth muscle differentiation (positive immunoreexpression of smooth muscle type actin and desmin) was revealed by dissecting the neoplastic cell nests. Differential microdissection from several tumor locations always confirmed the initial results. Excluding the possibility of significant contamination with host normal cells by careful and repeated sampling with microdissection, those findings could be the result of (1) true polyclonal tumors, which, because of an unfinished process of neoplastic cell selection, still show coexistent cell clones descended from different progenitor, or (2) an abnormal methylation of the HUMARA locus during tumor progression (hypermethylation of the active allele) expressing pseudopolyclonal patterns in true monoclonal neoplasms.

Another method used to investigate clonality, also based on the X-chromosome inactivation, depends on the mosaicism of protein expression in the normal tissues from heterozygous women showing two isoforms of the enzyme glucose-6-phosphate dehydrogenase (G6PD) [33-35]. The original report of clonality in medullary thyroid carcinomas and PCCs associated with multiple endocrine neoplasia relies on this technique [33,34]. The authors reported the presence of only one isoenzyme in tumor tissue from patients proven to be heterozygous for that marker and concluded that the initial mutation produces multiple clones of defective cells. Thereafter, each tumor arises as a final mutation in one clone of these cells. Essentially, the proposed hypothesis claims for an initial polyclonal and nonselected proliferation of defective cells, which then turns monoclonal through a process of cellular selection [36]. The histopathologic features in each stage have not been established, although theoretically adrenal medullary hyperplasia and PCC would represent the preneoplastic and neoplastic conditions, respectively.

Following the same methodology described for sporadic PCCs, HUMARA clonality assay was also performed with PCCs from members of an MEN-2a family [22]. The patients developed PCCs with no special histologic features, arisen on multinodular medullary hyperplasias. All except one tumor nodule revealed monoclonal methylation patterns, regardless of their sizes. The internodular adrenal medulla was required to show polyclonal methylation pattern in order to consider the case informative (criteria of case inclusion). Therefore, no conclusions could be obtained from the adrenal medulla with diffuse growth pattern. These results support the multistep tumorigenesis in the adrenal medulla of patients with MEN-2a [16,17,26]. An initial polyclonal stage would progressively evolve into monoclonal cell growths as the result of clone selection [6,36]. On the other hand, they also question the validity of nodule size as diagnostic criteria to distinguish hyperplasias from neoplasms. Tumor (or nodule) size is a time-dependent parameter and revealed a low specificity for case stratification by clonal pattern.
not be considered surprising for neoplastic processes with early transformation events, as those reported in most inherited tumor syndromes [23,24].

Evidence of Clonality Based on Tumor Genetic Alterations and Multistep Tumorigenesis

Although it is generally accepted the clonal evolution takes place in neoplastic transformation [1,6], some clonality studies have reported controversial results in early neoplasms and preneoplastic conditions of endocrine organs [37]. Several studies essentially confirm the multistep hypothesis for PCC tumorigenesis. Although the genetic mechanism of tumorigenesis has been referred to as different in pheochromocytomas and extra-adrenal paragangliomas [30], the same genetic targets seem to be involved in both sporadic and familial PCCs [26,29]. The general considerations mentioned above to analyze the malignant transformation and clonality [7] lead us to conclude that all these reports only provide information on clonal expansions in pheochromocytomas. The genetic homogeneity for a given marker would point to a kinetic advantage, provided by the marker itself or linked to it, that represents the basic mechanism of cell selection and tumor progression [1,6]. However, this isolated finding does not prove that all tumor cells come from the same progenitor and they are truly monoclonal. The same mutagenic event can affect cells from different progenitors (Fig. 4). Therefore, the descendant cells would share the same genetic abnormality (homogeneous pattern for a particular molecular marker, as expression of its clonal advantage and expansion), although they may have distinctive genetic background (different progenitors and true polyclonal origin).

Several tumor-suppressor genes have been demonstrated to play an important role in the above-mentioned clonal expansions of adrenal medullary neoplasms. As previously shown for other neoplasms, the tumor initiation and/or progression in PCCs involves multiple genes, mainly located by LOH analyses on chromosomes 1p, 3p, 17p, and 22q [26,29–31]. Some of these markers have also revealed significant association with clinicopathologic parameters, such as tumor volume (in the case of DNA deletions located on 1p, 3p, and 17p) [26] or with a distinctive transformation pathway (i.e., 1p34-36 and 3p25 deletions have been found in 45 and 56%, respectively, of PCCs, but not in extra-adrenal paragangliomas, whereas 3p21 deletions have been described in 50% of extra-adrenal paragangliomas, but not in PCCs) [30]. The relative incidence of each genetic alteration is quite variable from series to series, probably in relation to the limited number of cases analyzed. However, all series agree to show 1p deletions as the most frequent genetic finding, although no reference is available about its timing compared with other genetic abnormalities. That information would be especially valuable for the comparison between PCCs and adrenal medullary hyperplasias in order to determine its real nature either as an initiation-related genetic event or as a consequence of tumor progression.

On the other hand, the inherent genetic instability associated with neoplasms would explain the coexistence of several genetic abnormalities [1,6]. In that way, a significant association between interstitial deletions on 22q and 1p has been reported [29]. That finding suggests that the inactivation of multiple tumor-suppressor genes is required for PCCs development and progression (multistep tumorigenesis).
Considering tumor heterogeneity, an additional conclusion arises from that association. From the statistical point of view, homogeneous genetic alterations would show a low probability for the random association of two or more molecular markers in tumor cell populations from different origins (polyclonal tissues). Previous reports on LOH analyses, using Southern blot hybridization [38] and PCR-based techniques [39], have shown random and nonmalignant DNA deletions in 4–20% of normal tissues [40–42]. These percentages should be considered from two perspectives: (1) as a potential “confusing factor” in the analysis of case series, and (2) as the limiting threshold in order to interpret appropriately the biologic significance of any association between DNA deletions and tumor conditions. Heterogeneity is evident among histologically similar neoplasms from different patients (intertumor heterogeneity) and among different cells of the same neoplasm at a single time (intratumor heterogeneity), as well as at different points in time (tumor progression). Considering that variability, intratumor heterogeneity for LOH of multiple gene loci can be exploited as biparametric markers for the analysis of clonal selection in tumor progression that LOH genicity can be expression of either selective tumor evolution or simple passive byproduct of other mechanisms, such as genetic instability. The association of multiple genetic alterations would become statistically less probable as the number of molecular markers increases [43]. Then the random association of those markers would be better explained by a convergent selective process ending in the presence of a dominant clone [1,6]. That selection mechanism of cell clones with relatively homogeneous genetic constitution would represent the best approach currently available to define clonality based on tumor genetic markers. From this point of view, clonality could be considered the key element to understanding the endless biologic process of initiation and progression of tumors (cause and consequence at the same time) that also determines the otherwise linear natural history of neoplasms (Fig. 3).

Some experimental results (personal observations) support this point of view, based on the analysis of five polymorphic DNA regions (microsatellites) located on introns of four tumor-suppressor genes (p53, Rb, WT1, and NF1). Heterogeneous DNA loss of wild-type alleles (LOH analyses) was revealed in informative sporadic PCCs, involving p53 in 45%, Rb in 25%, WT1 in 44%, and NF1 in 50%. The comparative study of peripheral and internal tumor areas confirmed an increased accumulation of genetic deletions at the peripheral tumor compartment, expression of
both tumor progression, and multistep tumorigenesis. At that peripheral level, two or more genes showed LOH in agreement with the genetic instability of neoplasms. The low probability for random and simultaneous DNA deletion in nontransformed cell populations point to clone selection, ending in outgrowths of the "selected" clone, which becomes dominant and so-called monoclonal. The final tumor picture as monoclonal proliferation thus results from a selective process on a genetically heterogeneous cell population (Fig. 4).

Other factors also contribute to neoplastic development in endocrine organs. Somatic mutations of G-protein genes result in the constitutive activation of G-proteins and in an overall increase of the endocrine function. Along with the functional enhancement, proliferative advantages related to the trophic effect associated with the hormonal action have also been reported in these conditions [19]. That reason would contribute to explain the frequent association between overgrowth and hyperfunction of endocrine organs. G-proteins represent a key element of the intracellular signal transduction linking the extracellular ligands and the final cellular response. The active signal transduction normally present in most functional endocrine systems would explain the high sensitivity of those organs to abnormalities in that central pathway of signal transduction. That proliferative advantage would play an important role in the kinetic evolution and progressive selection resulting in cellular transformation in those disorders. Activating mutations at codon 201 of gsp have been found in different endocrine disorders, both neoplastic (pituitary adenomas, follicular thyroid adenomas, parathyroid adenomas, chemodectomas) and nonneoplastic (parathyroid or adrenal cortical hyperplasias) [19]. The same activating mutation has also been reported in primary and metastatic PCCs as well as in extra-adrenal paraganglioma from patients found to be wild-type at the germline DNA (extracted from leukocytes). That presence of concordant G-protein gene mutations in such a wide range of endocrine conditions and in different endocrine disorders in the same patient is consistent with a common underlying etiology.

Tumor Susceptibility and Clonality in PCC of Inherited Cancer Syndromes

The basic molecular mechanism is the same for both sporadic and inherited PCCs. However, the genetic background provides some valuable insights to understanding the general transformation process and the clonal evolution of tumors. The inherited cancer syndromes are essentially characterized by germline genetic abnormalities in certain DNA targets, frequently point mutations. Those abnormalities used to be activating point mutations targeting proto-oncogenes, such as ret in MEN-2 syndromes [32], or inactivating point mutations on tumor-suppressor genes, e.g. von Hippel-Lindau disease [18,28] or neurofibromatosis 1 [25]. In both cases (activating or inactivating mutations), the basic mechanism for the clonal expansion is the proliferative advantage provided by an appropriate genetic network (usually as additional somatic alterations on different genes) along with the increased susceptibility to neoplastic transformation (related with the germline mutation). Although a similar genetic background defines a given syndrome, different transformation pathways have been described in association with each tumor type and location. Therefore, variable relative frequencies of each genetic marker have been reported, i.e., using restriction fragment-length polymorphism analysis. 1p LOH has been found in all
PCCs associated with MEN-2 syndromes (67% including sporadic and Von Hippel-Lindau PCCs), whereas it was detected in only 13% of MEN-2-related medullary thyroid carcinomas [26]. The germline abnormality provides an increased susceptibility for tumor development that modulated by the cellular environment, will only end in neoplasms if multiple genetic loci are also involved (multistep tumorigenesis). Regardless of the predisposing genetic factor, chromosome 1p deletions are again the most frequently found molecular markers, as mentioned for sporadic PCCs (see Evidence of Clonality Based on Tumor Genetic Alterations and Multistep Tumorigenesis) [26,29,31].

The genetic background also modulates and determines the molecular mechanism involved in the transformation process. The most frequent inactivation mechanism of tumor-suppressor genes in PCCs involves both an inactivating point mutation in one allele (missense or nonsense mutations usually present as a germline defect) and the loss of the wild-type allele [26,27], analogous with the model proposed for different inherited malignant tumors [23,24,41,42,44,45]. However, additional inactivating mechanisms should be considered when no DNA loss of the wild-type allele is revealed. Such mechanisms include intragenic somatic mutations in the wild-type allele (also called homozygous inactivation) and hypermethylation of the tumor-suppressor gene as described for von Hippel-Lindau gene [28,46,47]. On the other hand, most of our knowledge on adrenal medullary hyperplasias comes from a restrictive group of pathologic conditions, such as experimental pathology (in vitro and in vivo studies) and, to a lesser degree, from human pathology (in the case of inherited cancer syndromes). Those factors make it more difficult to obtain reliable and clinically relevant conclusions from the molecular analyses, useful for general application. Although no established conclusions are available at the molecular level, the proliferative response of the adrenal medulla is better known, at least under specific and controlled conditions [49–52].

Current evidences suggests that chromaffin cell proliferation in adult rats is regulated by a combination of hormonal and neurogenic signals [49,50]. The adrenal medulla is innervated by several nerve fibers, which stimulate the secretion of catecholamines. Therefore, after chronic administration of a wide variety of catecholamine-depleting pharmacologic agents, such as the antihypertensive agent reserpine, the releasing of negative feedback
controls increases neurogenic stimulation on chromaffin cells [50]. The reflexively increased neurogenic stimulation of chromaffin cells is intended to meet the physiological needs of catecholamine synthesis, and would result in adrenal medullary hyperplasia and neoplasia. Catecholamine depletion would be compensated through mechanisms that normally adjust cell number, increasing the cell turnover rate and ending in chromaffin cell outgrowths [50,52]. Many strains of rats develop similar changes spontaneously in the course of aging. These models have revealed, using different methods (mitotic figure counting and incorporation of 5-bromo-2'-deoxyuridine into replicating nuclei), a significant increase in the proliferation indices after reserpine stimulation, which can be partially prevented by adrenal denervation [49,52]. Denervation also causes a significant decrease in the proliferation index in nonstimulated animals. This hyperproliferative stage is postulated as a prelude to neoplastic transformation. The chronic persistence of these signals and superimposed abnormalities would lead to the selection of specific cell clones, which becoming dominant, explain the development and progression of neoplasms [50].

Conclusions

The currently available evidence on tumorigenesis and clonality in the adrenal medulla can be summarized as follows:

1. Multistep tumorigenesis defines the evolution of pheochromocytomas, as evidenced by the presence of several genetic alterations.
2. Both the significant association of nonrandom genetic alterations (specially 1p and 22q interstitial deletions) and the topographic accumulation of genetic deletions at the peripheral tumor compartment support a convergent clone selection for these neoplasms.
3. Although many genetic loci show nonrandom abnormalities, the most frequently involved genes locate on chromosome 1p, regardless of genetic tumor background (sporadic or inherited predisposition).
4. Most pheochromocytomas should begin as monoclonal proliferations that do not always correlate with histopathologic features, particularly in inherited tumor syndromes.
5. Early histopathologic stages, described as adrenal medullary hyperplasias, are defined by hyperproliferative features in animal models and monoclonal patterns in the adrenal nodules from patients with MEN-2a.

References

Clonality in Pheochromocytomas


Bcl-2 Expression and DNA Fragmentation in Breast Carcinoma, Pathologic and Steroid Hormone Receptors Correlates

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B-cell leukemia/lymphoma (bcl-2) expression can override the apoptosis development in lymphoid and hormonally regulated tissue-like breast. The presence of estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR) have revealed in breast carcinomas, but they have not been correlated to the bcl-2 protein expression and DNA fragmentation markers. We evaluated the immunohistochemical expression of bcl-2 protein and hormonal receptors (ER, PR, AR) and differentiation grade in 37 infiltrating ductal carcinomas of the breast for which frozen tissues were available for DNA extraction. The immunohistochemical reaction for bcl-2 was considered positive if more than 50% of neoplastic cells had intense cytoplasmic staining, whereas for steroid receptor evaluation Batiffora’s criteria were used. The DNA was extracted according to the phenol-chloroform procedure and used for bcl-2 gene rearrangement study of the major breakpoint region (Southern blot) and for membrane-based end-labeling using digoxigenin-labeled nucleotides and E. coli DNA polymerase I (Klenow fragment). The results were quantified by three different observers. Low-grade carcinomas were positive for bcl-2 protein (27/28, 96.4%) and ER (15/28, 53.6%), whereas the remaining neoplasms were negative for bcl-2 (9/9, 100.0%) and ER (8/9, 53.6%) (p < 0.001). No statistically significant differences were revealed at the bcl-2, PR and AR comparisons. The Southern blot analysis for bcl-2 major breakpoint region showed neither rearrangement nor genetic amplification (densitometric study). Only the membrane-based end-labeling of DNA fragments showed correlation with bcl-2 protein and ER expressions. All except one bcl-2-negative tumor and two bcl-2-positive tumors had positive labeling using 7 pg of DNA at dot blot analysis (p < 0.002). The bcl-2 protein expression would allow both proliferation and cell progression by blocking apoptosis in well-differentiated, ER-positive breast carcinomas. In these neoplasms, DNA fragmentation as a molecular marker of apoptosis was prevented by bcl-2 expression.

**Key Words:** Bcl-2 expression—Steroid receptor—DNA fragmentation—Apoptosis—Breast carcinoma.


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**MATERIALS AND METHODS**

**Case Selection**

We used 37 consecutive cases of primary breast carcinomas from the files of the Tufts-New England Medical Center. Department of Pathology that also had frozen
tissue available. The tissues came from lumpectomy or mastectomy specimens and representative samples were prepared and stored in the tumor bank as previously described (22). Ten cryostat sections were cut in each sample, fixed in 3.7% formaldehyde phosphate-buffered saline (PBS), transferred to ice-cold methanol, then ice-cold acetone, and finally placed in a specimen storage medium and stored in a freezer (−10°C to −20°C) until staining. A frozen hematoxylin-eosin (H&E)-stained section of every case was used to document the presence of invasive neoplastic cells. All cases were infiltrating ductal carcinomas and graded according to the Bloom-Richardson system (7).

Immunohistochemical Detection of Bcl-2 Protein and Steroid Hormone Receptors

After rinsing in PBS the sections were incubated in 0.1% hydrogen peroxide in PBS for 10 min to quench endogenous peroxidase activity then rinsed in PBS. To suppress nonspecific binding of IgG, the specimens were incubated with 10% goat serum in PBS for 20 min (Vector Laboratories, Burlingame, CA, U.S.A.) before the addition of the following primary antibodies: bcl-2 (Oncogene Science Inc., Cambridge, MA, U.S.A., 10 μg/ml), androgen receptor (Novocastra Lab, Newcastle, U.K., 1 μg/ml), ER-ICA and PR-ICA (DAKO, Carpinteria, CA, U.S.A., 1 μg/ml each one). All primary antibodies were incubated overnight at 4°C.

After incubation with the primary antibodies, the preparations were rinsed in PBS, followed by incubation with biotinylated goat antimouse IgG 10 μg/ml (Vector Laboratories) for 45 min. A final 45 min incubation with Streptavidin-Horseradish peroxidase (Vector Laboratories) diluted 1:300 in PBS was followed by a PBS rinse. The reaction products were developed for 7 min with diaminobenzidine-tetrahydrochloride (DAB, Sigma, Chemical Co., St. Louis, MO, U.S.A.) 0.5% in PBS-hydrogen peroxidase 0.3%, followed by rinses in PBS, water, and a light hematoxilin counterstain.

In every case, negative and positive controls were simultaneously carried out. The negative controls consisted of consecutive tissue sections in which the primary antibodies were omitted. The positive controls were reactive lymph node (for bcl-2 protein), normal breast tissue (for ER and PR), and normal prostatic tissue (for AR) processed in the same way as the breast carcinoma samples. In addition, infiltrating lymphocytes served as internal positive controls for bcl-2 immunoreaction.

Interpretation of Immunohistochemical Labeling

The immunohistochemical reaction was evaluated on invasive neoplastic cells present in serial sections of the same block. Therefore, we could evaluate the same group of tumoral cells. In the evaluation of bcl-2 expression, the intensity and the number of positive cells were considered. At least 10 higher power fields from the most cellular area were evaluated. If more than 50% of invasive tumor cells showed an intense cytoplasmic staining then the case was scored as positive (Fig. 1). Infiltrating lymphocytes or benign breast epithelial elements in the same microscopic slide were used as positive internal controls.

For steroid hormone receptor analysis, the method proposed by Battifora et al. (4) was followed and a case was considered positive if ≥5% of the neoplastic nuclei in the invasive component showed distinct brown staining (Fig. 2).

DNA Extraction

A 0.25 mm³ sample of tumoral tissue was selected in each case for DNA extraction. The procedure was the organic extraction with phenol and chloroform, after cellular lysing (PBS—0.5% SDS) and protein digestion (Proteinase K, final concentration 0.5 mg/ml) and RNA digestion (RNase, final concentration 20 μg/ml), according to the manufacturer’s specifications (Oncor, Inc., Gaithersburg, MD, U.S.A.). The DNA was precipitated with ammonium acetate as conditioner (2 M final concentration) and two volumes of cold absolute ethanol, then resuspended in 150–200 μl of Tris-EDTA. The DNA was stored at 4°C until it was used.

The amount, concentration, and purity of the extracted DNA was evaluated by optical density (OD) measurements at 260 nm and 280 nm wavelength by means of a Beckman spectrophotometer. The OD ratio, at these wavelengths, is said to be an expression of DNA denaturation and fragmentation if RNA contamination can be excluded (our cases were all enzymatically digested by RNase) (3,29). The values of this variable for each case were used to study the presence of any relationship with those from quantitative end-labeling of DNA fragments by means of statistical regression analysis.

Gel Electrophoresis of Undigested DNA

Internaucleosomal fragmentation of DNA is widely utilized as the “hallmark” of apoptosis (1). In an attempt to detect the presence of DNA “ladders,” 10 μg of extracted DNA from each case was loaded in a 1.5% agarose gel to run for 2 h at 60 volts (20). The gel and electrophoresis buffer (Tris-acetic-EDTA) contained 0.5 μg/ml ethidium bromide, which allowed us to visualize the results of electrophoresis running under UV light.

End-labeling of DNA Fragments

In each sample, 6 μg of extracted DNA was taken to be labeled using a dNTP labeling mix (0.1 mM dATP,
FIG. 1. Intense cytoplasmic staining for bcl-2 on neoplastic cells in an infiltrating ductal carcinoma. Note the cytoplasmic immunoreaction on the infiltrating lymphocytes (positive internal controls). Frozen tissue, ABC-hematoxylin counterstaining, 450x.

FIG. 2. Positive nuclear staining for androgen receptor in an invasive ductal carcinoma. Frozen tissue, ABC, 450x.

0.1 mM dCTP, 0.1 mM dGTP, 0.65 mM dTTP, and 0.35 mM digoxigenin-11-dUTP, pH 7.5) (Boehringer-Mannheim GmbH, Germany) and 4 units of DNA polymerase Klenow fragment (Boehringer-Mannheim GmbH) in an appropriate buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 10 mM β-mercaptoethanol, and 0.01% bovine serum albumin) (Sigma Chemical Co., St Louis, MO, U.S.A.). The reaction was incubated at 37°C for 2 h. As positive control for end-labeling, 0.5 μg of pBR328 DNA was digested separately with BamH I, Bgl I, and Hinf I (Gibco-BRL, Gaithersburg, MD, U.S.A.) and then labeled under the same reaction conditions. As negative control all the reaction components except DNA were mixed together and incubated in the same conditions.

After end-labeling, the products were used for two purposes. First, one half was used for gel electrophoresis (1.5% agarose, 60 volts, 2 h) and subsequently blotted onto a positively-charged nylon membrane (Oncor), similarly as it will be described under Southern blot except for a transferring phase of 18 h. The membranes were washed in 0.1 M maleic acid, 0.15 M NaCl (Sigma Chemical Co.) and 0.3% Tween-20 (Fisher Scientific, NJ, U.S.A.), then the immobilized DNA were detected using antidigoxigenin alkaline phosphatase labeled antibodies (0.15 U/ml in 0.1 M maleic acid, 0.15 M NaCl pH 7.5 and 1% blocking reagent) (Boehringer-Mannheim GmbH) and stained for 3 min with the chromogen solution nitro blue tetrazolium chloride (0.45 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (0.175 mg/ml) (Gibco-BRL) in 0.1 M Tris-HCl pH 9.5, 0.05 M MgCl₂, 0.1 M NaCl (Sigma Chemical Co.). The reaction was stopped by washing the membrane for 5 min with 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0 (Sigma Chemical Co.).

Second, the other half was denaturated with 0.1 vol-
ume of 4 M NaOH. 100 mM EDTA at room temperature for 10 min and progressively diluted (5 times each step) in deionized water. Then 1 μl of every dilution was dot-blotted onto a positively-charged nylon membrane (Oncor) and the DNA immobilized by baking in an oven at 80°C for 2 h. The reaction development was the same described just above. As a positive control for the reaction, 1.5 μg of normal genomic DNA was simultaneously digested by EcoR I and BamH I (Gibco-BRL) and then taken to be labeled and diluted in the same way. As negative control, all the reaction components except DNA were mixed together and incubated in the same conditions. For each case, the greatest DNA dilution with a positive signal was recorded and its relative arbitrary value expressed as percentage of the greatest digested-control positive dilution.

Southern Blot

In every case, 10 μg of DNA was used for each restriction enzyme cutting (EcoR I, BamH I, Hind III in their appropriate buffer conditions) (Gibco-BRL) and incubated for 16 h at 37°C. Then the samples were loaded in a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide and run at 19 volts for 16 h. A picture was taken under UV light to check the results of electrophoresis running. As control, 10 μg of placental DNA were separately digested by each restriction enzyme and loaded into the gel.

After a light depurination (0.2 N HCl, 15 min), denaturation (1.5 M NaCl-0.5 N NaOH, 30 min), and neutralization (10X SSC, 45 min), the DNA was capillarity transferred to a positively charged nylon membrane using 10X SSC for 4 h. The DNA was immobilized on the membrane by baking at 80°C for 30 min.

The DNA samples were hybridized on the membrane using 1.5 ng/ml digoxigenin-labeled bcl-2 probe (Oncogene Science Inc.) at 68°C for 16 h in 10% dextran sulfate, 2.5X SSC, 1X Denhard solution, 10 μg/ml Poly(A) and 0.1% sodium dodecyl sulfate with 20 μg/ml of salmon sperm DNA as carrier (Oncor). The membranes were developed using antidigoxigenin alkaline phosphatase labeled antibodies (0.15 U/ml in 0.2% casein-PBS) (Boehringer-Mannheim GmbH, Germany) and the chemiluminescent substrate CSPD (0.12 mg/ml in 0.1 M diethanolamine, 1 measure MgCl2 0.02% sodium azide) (Tropix, Bedford, MA, U.S.A.). Several washings with the mixture 0.2% casein, PBS, 0.1% Tween-20 were used before and after antidigoxigenin antibody to avoid nonspecific reactions. The pictures were obtained, after 45 min of film exposure in a dark cassette, using a Kodak RP X-OMAT, Model M7B processor (Eastman Kodak Co., Rochester, NY, U.S.A.).

The developed film was used to measure the relative OD using a Beckman Instruments, Fullerton, CA, U.S.A.). In every case the values were expressed as an absorbance percentage of the respective control (they always were in the same film). As each case had three measurements corresponding to the three restriction enzyme used (EcoR I, BamH I, Hind III), the arithmetic average was selected as representative value.

Statistical Analysis

For qualitative variables the chi-square test with Yates’ correction and Fisher’s Exact Test was used to compare differences between groups. On the other hand, the analysis of variance (ANOVA) let us study any difference using Snedcor’s F-test when quantitative variables were studied. The difference was considered as statistically significant if p < 0.05 were obtained.

RESULTS

According to Bloom-Richardson criteria for grading breast carcinomas, a predominance of well-differentiated neoplasms were seen. In the immunohistochemical study, all except for one well-differentiated neoplasm revealed positive staining of bcl-2 on the cytoplasm: 28 (75.7%) of 37 neoplasms were 5–7 scored and 27 displayed intense bcl-2 staining. The remaining tumors (9/37, 24.3%) were moderately to poorly differentiated (Bloom-Richardson histologic score > 7) and showed weak-to-absent bcl-2 labeling and were rated as negative.

In the group of well-differentiated neoplasms, no tumors showed more than 10 mitotic figures/10 high-power fields (HPF) and around 50% of cases exhibited tubular formations (13/28, 46.4%) and low-nuclear pleomorphism (14/28, 50.0%). The moderate and poorly differentiated carcinomas showed a solid growth pattern without evidence of tubular lumina (9/9, 100%) and high-nuclear pleomorphism (7/9, 77.8%), whereas the mitotic rate was higher than 10 mitotic figures/10 HPF in 3 cases (33.3%).

The distribution of steroid receptors expression (all on neoplastic nuclei) in this series of breast carcinomas is shown in Table 1. The receptor most frequently expressed was AR (19/37, 51.4%) followed by ER (15/37, 40.5%) and PR (11/37, 29.7%). Moreover, a sharp difference in their expression could be seen. Most ER and PR staining was jointly presented (10 cases, 66.7% of ER-positive tumors and 90.9% of PR-positive tumors, 9 of them showing bcl-2 positivity and in 1 bcl-2-negative neoplasm), whereas AR was mainly found alone (10/19 AR-positive cases, 52.6%, 4 among bcl-2-positive tumors and 6 bcl-2-negative neoplasms).

The statistical study showed a close relationship between bcl-2 expression and grade of differentiation and ER status. Two main tendencies were disclosed: all bcl-2 positive tumors were well-differentiated neoplasms (p < 0.0001) and most of the bcl-2 negative carcinomas were
TABLE 1. Distribution of steroid hormone receptor reactivity according to bcl-2 expression in infiltrating ductal carcinoma of the breast

<table>
<thead>
<tr>
<th>Estrogen receptor</th>
<th>Progesterone receptor</th>
<th>Androgen receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>no.</td>
<td>(%)</td>
<td>no.</td>
</tr>
<tr>
<td>bcl-2 positive</td>
<td>14 (37.8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13 (35.2)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>bcl-2 negative</td>
<td>1 (2.7)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9 (24.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All but one were positive for two or more steroid receptors, five were positive for all three receptors.
<sup>b</sup> Eight cases expressed no steroid receptor (ER, PR, AR).
<sup>c</sup> The same case was positive for both ER and PR.
<sup>d</sup> Ten cases expressed AR alone, four bcl-2-positive tumors, and all six bcl-2-negative neoplasms.

ER negative (9 of 10 cases, p = 0.001). However, no statistically significant difference was shown between bcl-2 expression and AR status, although a weak tendency appeared to show AR nuclear positivity in bcl-2 negative carcinomas (6/10, 60%) and absence of AR nuclear staining in bcl-2-positive neoplasms (14/27, 51.9%).

After DNA extraction between 100 and 300 µg of DNA was obtained in each case with OD 260/280 ratios in the range 1.88–2.30. The OD at 260 nm wavelength shows the highest values for DNA, allowing us to calculate the DNA concentration, and therefore determine the exact amount of DNA for gel loading or enzymatic reaction.

The gel electrophoresis of undigested genomic DNA yielded a variable and continuous DNA smear reaching the 100–200 bp range, and the main portion of DNA at the upper gel zone above 50 kb in length (Fig. 3A). After end-labeling of DNA fragments the electrophoretic appearance showed nonsubstantial change (Fig. 3B): only three cases (two bcl-2-negative and one bcl-2-positive cases) had slighter intense bands of labeling at 3 kb range and another two (both bcl-2 positive) showed absent-to-mild labeling under 2 kb fragment size. The typically described “chromatin ladder” pattern was not found on the electrophoresis regardless of the end-labeling used. So, there was no significant qualitative differences on the DNA smears either for bcl-2 expression or for steroid hormone receptor expression.

The end-labeling of DNA fragment was arbitrarily quantified as end-labeling percentage of normal genomic DNA digested by restriction enzymes. The values were lower in the tumor groups positive for bcl-2 (average 3.39%, standard deviation 3.82) and for estrogen-progesterone receptors (average 3.12%, standard deviation 4.66) than in the tumor groups negative for bcl-2 (average 24.4%, standard deviation 66.59) and estrogen-progesterone receptors (average 14%, standard deviation 18.57), respectively. The ANOVA test showed statistically significant differences on quantitative end-labeling associated to bcl-2 and estrogen-progesterone receptor expressions (p = 0.01 and p = 0.015, respectively). No significant differences were observed in relation to androgen receptor expression.

The OD 260/280 ratio was recorded and the ANOVA test showed statistically significant differences (p = 0.01) between bcl-2-positive tumors (average 2.01, 0.09 standard deviation) and bcl-2-negative neoplasms (average 2.14, 0.16 standard deviation). No differences were found regarding steroid receptor expressions. The correlation between OD 260/280 ratio and quantitative end-labeling was done in two steps: first excluding those cases immunohistochemically negative for bcl-2 overexpression and then with all cases. For bcl-2 positive cases (Fig. 4A) a statistically significant linear regression could be obtained (as shown in the ANOVA test for linearity, Snedecor’s f = 27.16). When all cases were considered (Fig. 4B), a statistically significant linear regression only was yielded after logarithmic transformation of quantitative end-labeling (ANOVA for linearity, Snedecor’s f = 13.36).

The Southern blot study for the bcl-2 major breakpoint region always showed the presence of normal fragment size bands in the DNA after digestion by EcoRI (1.52 kb), BamH I (23 kb), and Hind III (4.2 kb) (Fig. 5). In all cases the signal intensity was slightly above 90% of the respective normal control (placental DNA digested by the same enzyme and simultaneously developed): an average of 91.3% (SD ± 0.15) for bcl-2 positive cases and 90.6% (SD ± 0.12) for bcl-2 negative cases. No statistically significant difference was obtain at the ANOVA test.

DISCUSSION

Most of the past studies on bcl-2 expression have focused on lymphoid tumors (21,32), although it has been also studied in nonhematolymphoid neoplasms, like prostate (10,19), breast (9,10), and neural or endocrine tumors (25,28). Previous results in hormone-controlled tissues, like breast or prostate, have shown a relationship between bcl-2 expression and hormonal function (6,9).

Using immunohistochemical methods bcl-2 protooncogene expression has been demonstrated in resting cells at the periphery of breast ducts in a normal gland.
(12). So, as the presence of bcl-2-positive cells in a tumor might be considered a near normal finding, we only scored as positive those cases expressing intense and broad cytoplasmic staining (intense labeling on 50% or more of infiltrating cells). This type of valuation should point out an overexpression of bcl-2 oncogene protein rather than retaining expression. We have to remember that previous reports (6,9) also defined the majority of bcl-2-positive carcinomas in those series demonstrated more than 50% positive cells.

This evaluation mode may explain the statistical results. The criteria used for our cases have shown a better...
discrimination for ER- and PR-negative breast carcinomas (only one bcl-2-negative case had positive ER and PR results). The aforementioned selective method includes in the bcl-2 positive group ER-negative tumors but a negative bcl-2 result points out a nonandrogen receptor negativity. This finding also suggests the possibility of estrogen and/or progesterone regulation of the bcl-2 protein expression (6,9). The absence of bcl-2 overexpression in moderate-to-poorly differentiated neo-

plasms would support the proposed role of other factors (eg., c-myc or p53) to maintain the long-term life expectancy of neoplastic cells and to allow the disease progression (14,17,26,30).

Similarly, Bhargava et al. (6) and Chan et al. (9) have reported a relationship between bcl-2 expression and ER positivity in their series of breast carcinomas. Bhargava et al. (6) found 24 bcl-2-positive cases of 41 breast carcinomas (58%). This incidence is lower than ours and
can be due to the differences in tissue preparations (their cases were paraffin-embedded), antibody reactivity, as well as the differences in criteria used for bcl-2 expression. The proposed ER-regulated bcl-2 expression may explain the finding of ER-positive cases with weak-positive bcl-2 expression (about 5% of cells) in the Bhar-gava's series (6). Therefore, the close association reported for bcl-2 and ER positive cases in the aforementioned series can be justified. Chan et al. (9) reported a low apoptotic index and mitotic index (lower than 6 mitotic figures/1000 cells) in bcl-2-positive breast carcinomas, most of them also expressing ER. In this way, those cases of low-grade malignancy would only need to grow a reduced cell lost from the proliferating neoplastic compartment, mechanism provided by preventing the programmed cell death when they expressed bcl-2 protein.

A different value should be noted from the AR results. The AR seems to have another way of regulation than ER and PR. In fact, it is usually expressed alone (10/19 AR-positive breast carcinomas showed immunoreactivity for neither ER nor PR) and, moreover, the expression of AR was slightly more frequent in bcl-2-negative cases (AR-positive tumors were 6/10 in the bcl-2-negative group and 13/27 in the bcl-2-positive group). These results may clarify the positive response to hormonal therapy in ER/PR negative tumors by crossactivation of the AR (13,15). Likewise, the long-term androgen ablation, as reported in the treatment of prostate carcinomas, would select bcl-2-positive cells not needing the trophic effect of androgen. Such a hypothesis is supported by the results reported by McDonnell et al. (19) where bcl-2 positivity was found in 10 of 13 androgen independent prostate carcinomas but in only 5 of 19 androgen-dependent tumors. Similar findings are reported by Colombel et al. (10) in their series. All metastatic hormone-refractory prostate carcinomas stained positive for bcl-2, whereas early stage hormone-responsive tumors showed positivity in 62% of cases.

Regarding grade of differentiation, a close correlation was found with bcl-2 positivity. All except one of our well-differentiated tumors showed an intense bcl-2 staining. In contrast moderate-to-poorest differentiated carcinomas were all negative for bcl-2. This correlation could help explain the close association between bcl-2 and tumor steroid receptor status. This finding agrees with those reported by Chan et al. (9), Bhargava et al. (6), and Rasbridge et al. (27). The first two case series yielded a predominance of well-differentiated carcinomas (like in the present study) with a bcl-2 positivity rate of 70.7% (29/41 cases) (9) and 58% (24/41 cases) (6). Rasbridge et al. (27) found bcl-2 positivity staining in only 26% (7/27) of pretreatment biopsies, but these were largely poorly-differentiated infiltrating ductal carcinomas (8/13 showed histologic grade III) with high mitotic indices.

The apoptosis development is associated with nuclear DNA digestion after endonuclease activation. It is said that the internucleosomal DNA cleavage and the DNA "ladder" pattern on gel electrophoresis characterize the apoptosis (2), although no case in this series showed the mentioned pattern. As a means of identifying apoptotic cell populations, detection of DNA "ladders" following agarose gel electrophoresis is second only to morphologic assessment in its applicability and utility (1). But we have to take into account other factors that can help us to explain the absence of DNA "ladder."

First, it has been reported in experimental studies the DNA ladder presence as a delayed finding associated to numerous morphologically evident apoptotic cells.
(31,36). However, we have to consider the DNA degradation associated to necrosis, especially for high-grade tumors, which provides DNA fragments of variable length and continuous smears on gel electrophoresis. This circumstance may partially hide the DNA-ladder finding if it is not prominent. Finally, the internucleosomal DNA cleavage is also related to the transcriptional chromatin activity: those active transcribing cells have a loose DNA-histone octamer connection (1,2,31). So, more DNA is exposed to endonuclease digestion beyond the internucleosomal region, especially for those bcl-2 negative cases showing prominent nucleoli and heterogeneous chromatin (related to a higher nuclear grade). For bcl-2-positive cases, the overexpression of this protein should block the bax-induced apoptosis when bcl-2/bax heterodimers formation takes place (24).

Nevertheless, the quantitative end-labeling revealed a close relationship to bcl-2 expression: the highest values were reached in the bcl-2-negative group. This finding suggests more DNA fragmentation in this tumor group as expression of apoptosis, necrosis or genomic instability in the neoplastic progression. Moreover, when we considered the DNA amount taken in the highest positive dilution at the end-labeling, all except one bcl-2-negative case showed positive labeling using less than one normal cell equivalent DNA content (6–7 pg. Fig. 4B). Different factors explain it: DNA fragmentation associated to a more extensive necrosis and apoptosis, as well as a higher DNA lability and sensitivity to extraction procedure in non well-differentiated tumors, probably in relation to genetic instability (18). Positive labels with less than one cell DNA content would mean either every cell carries fragmented DNA or a concentration of DNA fragmentation on a susceptible cell group. The latter explanation is supported by the heterogeneous pattern at the in situ end-labeling performed in some bcl-2-negative cases of this series (data not shown) as well as by the exponential relationship showed between quantitative end-labeling and OD 260/280 ratio. That exponential relationship is seen in any progressive process like the neoplastic cell growth in cell culture. According to the Novel’s hypothesis (23), a similar process can take place in the tumor cell during neoplastic progression (accumulative genetic damage with breakage and splicing) or cell death. This finding also represents an additional evidence of tumor heterogeneity.

For bcl-2-positive cases, the labeling levels and the linear relationship to the OD 260/280 ratio suggest a less frequent DNA fragmentation that affects different cells without cumulative effect. That finding may explain the less frequent apoptosis development and a long cell-life expectancy as well. The last situation also offers the opportunity to new genetic events conducting to an eventual progression.

The quantitative end-labeling was also statistically related to the ER-PR expression. This finding explains by means of bcl-2 overexpression most cases positive for ER and PR. In this way we cannot exclude a possible hormonal regulation of bcl-2 expression, especially in those well-differentiated tumors (6,9). This finding has also been confirmed in other studies (14,16,30). All these reports point out a close relationship between bcl-2 expression and favorable prognostic factors in breast carcinomas, especially ER expression (16,30) and lack of p53 protein expression (14,30).

The study of the bcl-2 gene by Southern blot showed no abnormalities: no evidence of gene rearrangement (presence of additional bands) affecting the major breakpoint region (the zone of highest instability) was revealed in this series. Moreover, the densitometric study of these bands when compared to their respective normal controls let us exclude gene amplification (the signal intensity in each case was always lower than that of normal control). These two findings suggest that bcl-2 overexpression (as it can be studied by immunohistochemical technique) would not be a primary event in the neoplastic breast transformation but a adaptive way used by the malignant cell as advantage survival.

In conclusion, bcl-2 overexpression seems to be an advantage mechanism of the neoplastic cells to reach long-term growth in well-differentiated ER-positive breast carcinomas. The ER/PR-negative high-grade tumors, in contrast, should not need that way to persist and even progress. In the latter situation the neoplastic cells can be partially sustained by the AR pathway (although with a nonstatistically significant difference in the present study).

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Aggressive Fibromatosis (Desmoid Tumor) is A Monoclonal Disorder

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Aggressive fibromatosis (also called deep fibromatosis or desmoid tumor) is a proliferation of cytologically benignappearing fibrocytes, often resulting in significant functional loss. The nature of the lesion is controversial: some evidence suggests that it is a reactive process, whereas other evidence supports a neoplastic etiology. The pattern of X chromosome inactivation, using a technique based on polymerase chain reaction (PCR) amplification of a hypervariable CAG repeat region flanking Hhal restriction sites of the human androgen receptor gene, was determined in four cases in which cryopreserved tumor and adjacent normal tissue were available. All four tumors demonstrated a monoclonal pattern, while the adjacent normal tissues demonstrated a polyclonal pattern. This demonstrates that aggressive fibromatosis is proliferation of cells derived from a single clone with a growth advantage, and thus is likely a neoplastic process.

Key Words: Aggressive fibromatosis—Desmoid—Clonality—Polymerase chain reactions.


Aggressive fibromatosis (also called fibromatosis or desmoid tumor) is a rare lesion in which there is proliferation of cytologically benign fibrocyte-like cells that invade surrounding structures. Treatment for this lesion is difficult, as it frequently recurs after surgical excision and can result in significant functional loss. Radiation therapy is effective in controlling growth, but chemotherapeutic modalities are effective only in select cases (2,9).

Previous studies show that aggressive fibromatosis expresses several proliferative growth factors (2,3). Cytogenetic abnormalities, including trisomy 8, trisomy 20, or absence of 5q, are present in some cases (5,6,7). It does not contain somatic mutations in the suppressor genes Rb or P53 (9,12) or in the signal transducer, Gs (4). Patients with familial polyposis coli have a germline mutation of the APC gene in one allele. Some of these patients develop abdominal desmoids (histologically identical to the aggressive fibromatoses), and these are associated with somatic mutation in the other APC allele (11,15,16).

There is controversy about the nature of the lesion, with some evidence pointing to a neoplastic disorder and other evidence suggesting a reactive process. Aggressive fibromatosis shares histologic similarities with hypertrophic scar (a reactive process), with identical cytology of the fibrocyte-like cells (9). Its clinical behavior, however, suggests a neoplastic process, with lower recurrence rates following surgical treatment with wider surgical margins, and occasional response to antineoplastic agents (2).

Neoplasms derive from a single progenitor cell with a growth advantage, and thus are monoclonal (8). Reactive processes exhibit proliferation of multiple cells and are polyclonal. One X chromosome is inactivated in each cell in females during embryogenesis (lyonization). A monoclonal process will exhibit inactivation of the same X chromosome in all the cells, while a polyclonal proliferation exhibits random X chromosome inactivation (17).
One technique for determining X chromosome inactivation uses the human androgen receptor gene, located on the X chromosome (1). This gene contains a hypervariable CAG repeat region adjacent to the 5' side of an Hhal restriction site. The variability of the repeat has a 90% chance of being of different length between two parents. DNA methylation of the inactive chromosome renders Hhal unable to cut the DNA. Polymerase chain reaction (PCR) amplification of the DNA segment containing the hypervariable repeat and the restriction site will identify the two alleles, which usually are of different length. Pre-digestion with Hhal allows amplification of only the inactive X chromosome. A monoclonal proliferation will contain a single-sized amplification product after Hhal digestion or a skew towards a single-sized PCR amplification product, compared with the pattern of undigested DNA. A polyclonal proliferation will contain equal amounts of PCR amplification products of both sizes after digestion, as there is random X chromosome inactivation, with the same pattern in digested DNA, undigested DNA, and normal tissue (13,14,18).

The purpose of this study was to determine the pattern of clonality in aggressive fibromatosis, thus determining whether the lesion is a neoplastic proliferation or a reactive process. Both tumor tissue and adjacent normal tissues were studied using a human androgen receptor gene based assay.

METHODS

Cases were obtained from the tumor bank of the New England Medical Center. Fresh tissue was obtained at the time of surgical excision and cryopreserved as soon as possible after surgery. In selected cases, normal tissues (negative resection margins) were also cryopreserved as controls.

Search of the tumor bank revealed four cases in female patients in which tumor and normal tissues were available. The histology of the cases and clinical records were reviewed to confirm the diagnosis. Two lesions were of the upper extremity and two were of the lower extremity. None of the individuals had undergone previous chemotherapy or radiotherapy. The lesions had been operated on for recurrence after previous surgical treatment. None of the lesions were superficial (e.g., were not Dupuytren disease).

The frozen tissue was divided; one section was used for DNA extraction and the other for histologic evaluation. Samples for histology were embedded in OCT, and sections were prepared with a cryostat and stained with hematoxylin and eosin.

The clonality assay was performed as previously described (13). Each sample was tested in duplicate. DNA was extracted using proteinase K digestion, ethanol precipitated, and resuspended in Hhal buffer. The DNA was divided into two identical aliquots of 0.5, 1.0, or 2.5 μg each, one of which was digested with Hhal overnight at 37°C. A small aliquot of digested and undigested DNA was electrophoresed on an agarose gel along with the products from simultaneously performed digestion, of linearized OX174 to verify complete digestion. Following the digestion, DNA from both aliquots underwent phenol/chloroform treatment. The polymerase chain reaction was used with 0.3 μM each of previously described oligonucleotide primers (CCAGGAGCTTCTCCA-GAAC and TACGATGGGTTGGAGAAA) (13); a 1.5 mM MgCl2 buffer; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; and 100 nM amounts of 32P-labeled TTP (800 μCi/ml). A “hot” start was performed (97°C for 5 min, followed by 85°C for 10 min), followed by three cycles with a prolonged denaturing time (95°C for 4 min; 55°C for 45 sec; and 72°C for 30 sec), and 23 regular cycles with a denaturing time of 30 sec. PCR products were electrophoresed on an 8% polyacrylamide gel overnight at 30 volts, and autoradiography performed for 48 h with an intensifier.

RESULTS

Histology verified the typical appearance of aggressive fibromatosis in all of the lesional tissue (Fig. 1). The adjacent normal tissue consisted of normal-appearing fascia in two cases and normal-appearing muscle in two cases.

All four of the tumors demonstrated a significantly skewed pattern after DNA digestion (Fig. 2). The differences were readily apparent to visual examination, as illustrated in Fig. 2, and densitometry was not necessary. All four patients had a different length of the human androgen receptor gene from the two alleles, and the data from each patient were informative. Three cases exhibited inactivation of the chromosome containing the smaller-sized gene, and one case showed inactivation of the larger-sized fragment. The surrounding normal tissues demonstrated the same pattern with and without digestion. Thus, a monoclonal pattern was identified in the tumor tissues, whereas a polyclonal pattern was identified in the adjacent normal tissues.

DISCUSSION

There was a clear monoclonal pattern of X chromosome inactivation in these cases. Although a light second band was apparent in the tumor samples subjected to endonuclease digestion, the difference in
band densities was not subtle to visual inspection. The small amount of signal from the second band likely represents normal cells mixed with the tumor.

Previous studies show that this assay used to detect X chromosome inactivation is quite reliable for most cases (1,13,14,18). The technique is not informative in cases in which both alleles contain genes of equal length, or when normal tissue demonstrates a skewed pattern of inactivation. The clonality assay can be utilized with paraffin-embedded material (13); however, high-quality DNA is needed for this assay, and this can be more readily obtained from cryopreserved tissues. The use of paraffin-embedded samples would give us a larger sample size, but as all of our patients gave informative data, we did not believe that additional data from paraffin samples were necessary. The polyclonal pattern of the adjacent normal tissues strengthens our findings. Other studies showing clonal cytogenetic abnormalities (5–7) and X chromosome inactivation (10) in aggressive fibromatoses further supports our results.

Similar techniques based on other genes located on the X chromosome can be used to determine clonality, such as the phosphoglycerate kinase gene or the hypoxanthine phosphoribosyltransferase gene. However, these sites do not have the degree of heterozygosity as the human androgen receptor locus, and thus they are not informative in as large a percentage of individuals (18).

The monoclonal nature of aggressive fibromatosis demonstrates that it derives from a single progenitor cell with a growth advantage. Aggressive fibromatosis is not composed of normal fibrocytes stimulated by proliferative growth factors, nor does chemotaxis, recruiting surrounding fibrocytes, likely play a key role in its pathogenesis. Identification of a monoclonal proliferation may be helpful in distinguishing aggressive fibromatosis from hypertrophic scar, but further study of the clonality characteristics of scar is necessary. The knowledge that this is a monoclonal lesion will help to focus future research on the underlying abnormality responsible for the cell’s growth advantage.

FIG. 1. Histologic study of an aggressive fibromatosis. Spindle-shaped cells, without mitoses, in a collagenous matrix are illustrated (×100).

FIG. 2. Results of clonality assay from one case of aggressive fibromatosis and surrounding normal tissues. Lanes labeled D are amplicons from DNA digested with HhaI. Lanes labeled U are loaded with amplicons from undigested DNA. Lanes labelled D are loaded with PCR amplification products from 0.5 µg of starting DNA, while lane D1 is loaded with product from 1.0 µg starting DNA and D2 is loaded with product from 2.5 µg starting DNA. Tumor lanes are DNA from aggressive fibromatosis, and normal lanes are loaded with DNA from surrounding normal fascial tissue from the same patient. Two different-sized products of the human androgen receptor gene are identified. HhaI digestion resulted in a single-sized product for aggressive fibromatosis, but two products for the surrounding normal fascia, indicating a monoclonal pattern of the tumor but a polyclonal pattern of the surrounding normal tissue. The lack of a second band in lanes D1 and D2 strengthens this finding.
REFERENCES


Short Communication

Increased β-Catenin Protein and Somatic APC Mutations in Sporadic Aggressive Fibromatoses (Desmoid Tumors)

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Sporadic aggressive fibromatosis (also called desmoid tumor) is a monoclonal proliferation of spindle (fibrocyte-like) cells that is locally invasive but does not metastasize. A similarity to abdominal fibromatoses (desmoids) in familial adenomatous polyposis and a cytogenetic study showing partial deletion of 5q in a subset of aggressive fibromatoses suggests that the adenomatous polyposis coli (APC) gene plays a role in its pathogenesis. APC helps regulate the cellular level of β-catenin, which is a downstream mediator in Wnt (Wingless) signaling. β-Catenin has a nuclear function (binds transcription factors) and a cell membrane function (is a component of epithelial cell adherens junctions). Six cases of aggressive fibromatosis of the extremities from patients without familial adenomatous polyposis, or a family history of colon cancer, were studied. Immunohistochemistry, using carboxy and amino terminus antibodies to APC, and DNA sequencing showed that three of the six contained an APC-truncating mutation, whereas normal tissues did not contain a mutation. Western blot and Northern dot blot showed that all six tumors had a higher level of β-catenin protein than surrounding normal tissues, despite containing similar levels of β-catenin mRNA. Immunohistochemistry localized β-catenin throughout the cell in tumor tissues, although it localized more to the periphery in cells from normal tissues. Reverse transcription polymerase chain reaction showed that the tumors expressed N-cadherin but not E-cadherin (a pattern of expression of proteins making up adherens junctions similar to fibrocytes), suggesting that the specific adherens junctions present in epithelial cells are not necessary for β-catenin function. Increased β-catenin may cause the growth advantage of cells in this tumor through a nuclear mechanism. The increased protein level, relative to the RNA level, suggests that β-catenin is degraded at a lower rate compared with normal tissues. In some cases, this is caused by a somatic mutation resulting in a truncated APC protein. (Am J Pathol 1997, 151:329–334)

Aggressive fibromatosis occupies an unusual position in the progression of neoplasia, as its cells have lost local control of growth but do not have the capability of forming metastases. It is one of a group of clinically heterogeneous disorders composed of cytologically similar spindle-shaped (fibrocyte-like) cells termed fibromatoses.¹ The nomenclature of the fibromatoses is not uniform, and terms such as aggressive fibromatosis, deep fibromatosis, and desmoid tumors are all used to describe the same lesion. Aggressive fibromatosis is the most invasive of the fibromatoses; it infiltrates into surrounding structures, frequently recurs after surgical treatment, and often results in significant loss of function. The lesion causes loss of function due to impingement on surrounding structures and potentially causes mortality if it involves vital structures. Current treatments include chemotherapy, which is effective in only some cases, and radiation therapy, which is effective in many cases but associated with significant side effects. Operative resection with wide margins are needed to surgically eradicate the tumor, and occasionally it is necessary to perform an amputation for a tumor involving an extremity.²–⁴ Study of clonality of aggressive fibromatosis shows that it is a

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monoclonal proliferation. As it is due to a clone with a growth advantage, it is possible that a mutation increasing cell proliferation is responsible for its pathogenesis.

Ten to fifteen percent of patients with the familial cancer syndrome, familial adenomatous polyposis (FAP), develop abdominal fibromatoses (usually termed abdominal desmoids) associated with a germ-line mutation in one allele and somatic mutation in the other allele of the adenomatous polyposis coli (APC) gene. Somatic APC mutations in abdominal fibromatoses in patients with FAP tend to be located between codons 1309 and 1450, in an area termed the mutational cluster region. They result in an early stop codon and a truncated protein product. Cytogenetic study of aggressive fibromatoses from patients without FAP show a number of anomalies, with trisomy 8, trisomy 20, and partial loss of the long arm of chromosome 5 occurring most frequently. The occurrence of aggressive fibromatoses in FAP, along with the cytogenetic demonstration of loss of a portion of 5q in some lesions in patients without FAP, suggests an APC mutation in some spontaneously occurring aggressive fibromatoses.

APC binds β-catenin, a protein involved in Wnt signal transduction. Wnt signaling in humans is homologous to the Wingless signaling pathway in Drosophila and functions in normal development. Wnt signaling is also activated in some human malignancies, likely resulting in cell proliferation. Wild-type APC binds β-catenin and, when β-catenin is present at high concentrations, binds to the serine-threonine kinase, GSK3β. GSK3β phosphorylates APC, activating a second binding site for β-catenin, resulting in β-catenin degradation. APC truncations due to early stop codons in the mutational cluster region may lack the GSK3β binding site, resulting in a higher β-catenin level. Wnt expression results in increased β-catenin, potentially due to decreased GSK3β activity. β-Catenin is a key mediator in Wnt signalling, and, in Drosophila, beta-catenin mimics Wnt activity. The β-catenin protein level can also be increased by mutations in β-catenin itself that interfere with normal protein degradation. Thus, there are a number of mechanisms resulting in an increased β-catenin level, including Wnt activation and APC truncation.

β-Catenin binds nuclear transcription factors in the lymphoid enhancer-binding factor-1 (LEF-1) family. LEF-1 is an architectural binding factor, causing a bend in DNA, allowing other nuclear proteins to bind. β-Catenin bound to LEF-1 causes a bend in DNA that may be different than the bend formed by LEF-1 alone. Although LEF-1 is expressed only during development and by adult lymphoid tissues, other members of this family, Tcf-1, -2, and -4, are expressed by colonocytes. β-Catenin is also a member of the cell membrane adhesion junctions. These junctions play a role in cell-cell communication and cell adhesion. In epithelial cells, during wounding, or when tumors metastasize, the junctions disassociate. The disassociation and association of adherens junctions is hypothesized to mediate the contact inhibition signal. Proteins making up adherens junctions, such as E-cadherin, act as tumor invasion suppressors, with more invasive tumors lacking the protein. APC competes with E-cadherin for binding to β-catenin, therefore, has potential functions in cell adhesion and in nuclear transcription.

There is a higher frequency of colonic neoplasms than tumors elsewhere in FAP, suggesting that APC truncation plays a role in only select cell types. This could be due to differences in expression of APC or differences in how APC functions in different cell types. Differences in expression could be related to alternative splicing of the 5' noncoding portion, although all alternative sequences, except for one found in the brain, are expressed by a wide variety of tissues. Differences in cell type may be related to differences in adherens junctions. The quantity and protein content of adherens junctions differs between cell types, with fibrocytes having a smaller number compared with colonocytes, containing N-cadherin rather than E-cadherin.

We aimed to determine whether β-catenin protein is elevated in sporadic aggressive fibromatoses compared with surrounding normal tissues and whether somatic mutations causing APC truncations are present in a subset of the tumors. In addition, we will determine the expression of E-cadherin and N-cadherin (to determine whether this tumor contains epithelial or fibroblast-like adherens junctions) and localize the site of β-catenin in the cell to determine whether β-catenin more likely acts through a cell membrane or nuclear function.

**Materials and Methods**

Six cases of aggressive fibromatoses of the extremities were studied. All were deep tumors of the extremities (two of the upper and four of the lower extremity). The patients had isolated tumors without evidence of colonic polyps and without a family history of colonic neoplasia or FAP. Samples were obtained from the initial operative procedure before undergoing chemotheraphy or radiation therapy. Surrounding normal tissue was also obtained for study and processed in an identical manner. Tissue was snap-frozen as soon as possible after surgical excision and stored in liquid nitrogen. In four cases, tissue was also cryopreserved in OCT before storage in liquid nitrogen. Medical records, radiographs, and formalin-fixed, paraffin-embedded materials were available to confirm the diagnosis. Colon cancers containing an APC mutation and the SW480 colon cancer cell line (American Type Culture Collection, Rockville, MD), which contains an APC-truncating mutation, were used as positive controls.

**Immunohistochemistry**

OCT-embedded cryopreserved tissues were cut into 12-µm sections and fixed in 0.3% hydrogen peroxide in absolute methanol at room temperature for 30 minutes. Frozen sections from the additional cases were embedded in OCT and then sectioned. A sporadic colon cancer with an APC mutation and sections of normal colonic mucosa from the same patient were used as positive and negative controls and were processed along
with the fibromatosis tissue. The sections were washed in PBS, blocked with 1% horse serum for 30 minutes at room temperature, and washed again in PBS. The sections were incubated with monoclonal antibody to the amino terminus of APC (APC-3, Oncogene Science, Cambridge, MA) or the carboxy terminus of APC (APC-4, Oncogene Science) overnight at 4°C. Additional sections were incubated in control mouse immunoglobulins. Protein digestion was performed using Pronase, 50 μg/ml, for 5 minutes at room temperature for the APC-4 antibody before incubation to enhance antigen detection, after initial experience with the colon carcinoma and normal mucosa. Antibodies were used at 1.0 μg/ml, 1.5 μg/ml, and 2.0 μg/ml. The tissues were washed with PBS and incubated with a secondary anti-mouse immunoglobulin and detected using immunoperoxidase staining (Vector Laboratories, Burlingame, CA). Hematoxylin was used as a counterstain.

Immunohistochemistry for β-catenin was performed on paraffin-embedded, formalin-fixed materials. A previously published microwave retrieval technique was used. Briefly, tissues were dewaxed and quenched in a hydrogen peroxide/methanol solution, immersed in 10 mmol/L citric acid, and heated 15 minutes in a 750-W microwave oven. After washing and blockade using 1% bovine serum albumin, slides were incubated with a monoclonal anti-β-catenin antibody (Transduction Laboratories, Lexington, KY) at 10 μg/ml overnight at 4°C and detected using the same technique as for APC. Hematoxylin was used as a counterstain.

**DNA Analysis**

DNA was extracted from cryopreserved tissues using proteinase K. The mutational cluster region of APC occurs within exon 15, and this exon was chosen for sequencing. Exon 15 was amplified using polymerase chain reaction (PCR) and a series of 20 overlapping primer pairs. An aliquot of each PCR product was electrophoresed, stained with ethidium bromide, and observed under ultraviolet light to ensure that a single product of appropriate size was amplified. Additional aliquots were purified using the QIAquick PCR purification column (Quiagen, Chatsworth, CA) and sequenced using an automated sequencer, based on the PCR. The PCR primers were used as the primers for sequencing.

**RT-PCR**

RNA was extracted using guanidinium isothiocyanate homogenization followed by cesium trifluoride ultracentrifugation. mRNA was converted to cDNA using reverse transcriptase with a poly(T) primer. PCR was performed using specific oligonucleotide primers for GAPDH, E-cadherin, N-cadherin, and select primer pairs for exon 15 of APC. RNAse-treated controls were also used. Primers and conditions for GAPDH and E-cadherin were previously published. The upstream primer for N-cadherin was selected from base pairs 80 to 100 (ATAGAGATAAAAACCTTCA) and downstream primer from base pairs 479 to 498 (ATTGTGATTGTAACATGT). An annealing temperature of 50°C was used. The resultant products were electrophoresed, stained with ethidium bromide, and photographed under ultraviolet light.

**Northern Dot Blot**

Equal amounts of extracted total RNA (4 μg) from each sample, and from control cell lines, were prepared on a nylon membrane for dot blot. Probes for β-catenin and GAPDH were digoxigenin labeled from specific cDNA (available from American Type Culture Collection) using the Dig Easy Hyb kit according to the manufacturer’s specifications (Boehringer Mannheim, Laval Quebec, Canada). Hybridization was carried out overnight at 50°C and detected using an anti-digoxigenin antibody and chemiluminescence (Boehringer Mannheim). Northern blot using a larger quantity of RNA (15 μg) was also performed on one sample to verify the dot blot results.

**Western Blot**

Protein was extracted from tissue samples by homogenization in lysis buffer (1% SDS, 10 mmol/L Tris/HCl, pH 7.4), followed by 10 seconds of microwaving and 5 minutes of centrifugation (12,000 X g). Equal amounts of total protein were electrophoresed on an SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and stained to verify an equal amount of transferred protein from each sample. Western blot was performed using a monoclonal antibody to amino acid residues 571 to 781 of β-catenin (Transduction Laboratories). Hybridization was carried out overnight at 4°C and detected using an anti-mouse IgG-horseradish peroxidase secondary antibody and chemiluminescence.

**Results**

Immunohistochemistry showed the amino-terminal portion of APC present in tumor tissue, adjacent vascular tissue from the same slides, and normal tissues. The antibody to the carboxyl-terminal portion of the protein stained the cytoplasm of the vascular and normal tissue cells but was absent in the aggressive fibromatosis cytoplasm (Figure 1) in two cases. In two additional cases, there was staining using both the carboxyl-terminal and amino-terminal antibodies. The remaining two cases did not have tissues cryopreserved in OCT available, and the poor preservation of cellular morphology made the results impossible to interpret. Two of the four cases with reliable data contained a truncated protein.

Sequencing data (Figure 2) for exon 15, which contains the mutational cluster region, showed mutations resulting in an early stop codon in three of the six cases. Two cases showed loss of heterozygosity, with the mutation present in both alleles. The third case demonstrated two base substitutions at codons 1492 and 1493, with a mixture of wild-type and mutant sequences observed. Normal tissue DNA from all three
cases demonstrated only the wild-type sequence. One case showed a frameshift mutation in both alleles at codon 1324 (tumors demonstrate CCCA; wild-type sequence is CCA), resulting in a downstream stop codon (TAG) at codon 1331. The second case demonstrated a frameshift mutation in both alleles at codon 1371 (AT for AGT) with a downstream stop codon (TAA) at 1414. The third demonstrated a substitution of T for G in one allele causing a stop codon (TAA) at 1493. The remaining three cases demonstrated the wild-type sequence. The cases demonstrating a frameshift mutation with loss of heterozygosity showed immunohistochemical staining to only the amino-terminal antibody, whereas two of the three cases with a wild-type sequence showed staining with antibodies to both the carboxy and amino termini. The remaining two cases did not have adequately preserved tissues available for immunohistochemistry.

Western blot showed that all six cases exhibited increased β-catenin protein relative to adjacent, normal, fibrous tissues (Figure 3). Northern dot blot showed a similar level of β-catenin RNA. The increased protein level, without increased transcription, suggests decreased protein or mRNA degradation. Immunohistochemistry for β-catenin (Figure 1) localized β-catenin in the tumor tissue throughout the cell, whereas in adjacent normal tissues, β-catenin was localized principally in the cell periphery. This localization pattern was the same for tumors containing an APC truncation and for tumors with a wild-type APC sequence.

Reverse transcriptase PCR demonstrated that the fibromatoses expressed N-cadherin but not E-cadherin, whereas the SW480 colon cancer cell line expressed E-cadherin but not N-cadherin (data not shown). Thus, aggressive fibromatosis expresses the same proteins making up adherens junctions as fibrocytes. All of the cases expressed RNA for exon 15 of APC. The case with both a mutant and wild-type sequence identified also expressed both the mutant and wild-type sequences.

**Discussion**

All of the tumors exhibited increased β-catenin protein compared with normal tissues, despite containing similar levels of mRNA, suggesting that tumors exhibit de-
The fibroproliferative lesions in the tumors compared with normal tissue despite similar mRNA levels is illustrated in all cases. SW 480 is loaded with protein and mRNA from the SW480 colon cancer cell line. The increased protein level in tumors is likely due to decreased degradation.

There are a number of potential roles for β-catenin in neoplasia. β-Catenin is present in normal cells, and in some epithelial cell tumors, it is present at decreased levels compared with normal cells. This decrease is thought to play a role in increased cell mobility by disrupting adherens junctions. In contrast, β-catenin may be elevated in other tumors, such as by APC truncation in colonic neoplasia, by mutations in β-catenin itself, or by Wnt activation. Expression of at least one Wnt is demonstrated in some breast, lung, and prostate carcinomas and some melanoma. Wnt is also expressed at high levels in the benign breast lesion fibroadenoma. Although Wnt activation is not demonstrated by our data, the elevated β-catenin levels and Wnt expression in fibroadenoma suggest a common role for Wnt signaling in fibroproliferative lesions.

Somatic mutations causing APC truncations are also present in a subset of another locally invasive sporadic lesion, colon polyps. In both aggressive fibromatosis and colonic polyps, the APC truncation is likely responsible for the local growth of the tumor cells, as transfection of APC into cells decreases proliferation. There are clinical differences between these two lesions. For instance, unlike aggressive fibromatosis, colonic polyps containing APC truncations progress to malignancy. Another difference between the two lesions is the type of adherens junction present, as aggressive fibromatosis expresses N-cadherin rather than E-cadherin. Thus, the specific epithelial cell adherens junctions are not necessary for APC truncation to allow cell proliferation. The localization of β-catenin throughout the cell in tumor tissues suggests a nuclear role as opposed to a cell membrane role. Thus, APC truncation and elevated β-catenin likely function to alter proliferation through a nuclear mechanism.

Demonstration of somatic APC mutations in one allele with germline mutations in the other allele in abdominal fibromatoses in individuals with FAP gave evidence that the Knudson hypothesis holds true for APC truncation to cause cell proliferation in fibroblasts. Despite this, the majority of evidence from colonic neoplasia supports a dominant negative role for APC truncation. Evidence for this is the correlation of site of mutation with disease severity in FAP. The occurrence of mutations in a single allele in sporadic colonic neoplasms (tumors exhibit either a loss of heterozygosity or a mutation in only one allele). The demonstration that APC forms a dimer of wild-type and mutated protein. Unlike the abdominal fibromatoses in FAP, one of the sporadic tumors exhibited both a mutant and wild-type sequence, presumably resulting in both a truncated protein and a full-length product. The two sequences are most likely due to the mutation occurring in only one allele, with a wild-type sequence to the other allele. An alternative explanation for this sequencing result is that there are normal cells within the sample studied. As aggressive fibromatosis is a monoclonal disorder, the tissue studied should be homogeneous and, thus, less likely to contain normal cells. APC has the potential to act in a dominant negative fashion in aggressive fibromatosis. A mutation in a single allele alone, however, is not sufficient to cause aggressive fibromatosis. If this were true, then all individuals with FAP would have the tumor, whereas approximately 10% exhibit the lesion.

Elevated β-catenin protein, caused by a somatic APC mutation resulting in a truncated protein in some cases, is present in sporadic aggressive fibromatosis. β-Catenin protein functions through an epithelial cell adherens-junction-independent mechanism. The localization of β-catenin protein throughout the cell (including the nucleus) further suggests a nuclear role for the elevated β-catenin protein. Somatic mutations resulting in a truncated APC increase cell proliferation and potentially act in a dominant negative manner in fibrocytes as well as epithelial cells. Current treatments for aggressive fibromatosis are less than satisfactory. Adjuvant treatments based on manipulation of cellular growth control may improve the outcome for individuals with this disorder. Perhaps a treatment based on modulation of the function of a truncated APC, or elevated β-catenin, by manipulating a downstream mediator could be devised.

References

E-cadherin and Adenomatous Polyposis Coli Mutations Are Synergistic in Intestinal Tumor Initiation in Mice

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See editorial on page 1161.

Background & Aims: Inactivation of the adenomatous polyposis coli (APC) gene is observed at early stages of intestinal tumor formation, whereas loss of E-cadherin is usually associated with tumor progression. Because both proteins compete for the binding to β-catenin, an essential component of the Wnt signaling pathway, reduction of E-cadherin levels in an Apc mouse model could influence both tumor initiation and progression. In addition, loss or haploinsufficiency of E-cadherin may affect tumorigenesis by altering its cell-adhesive and associated functions.

Methods: Apc1638N mice were bred with animals carrying a targeted E-cadherin knock-out mutation.

Results: Double heterozygous animals showed a significant 9-fold and 5-fold increase of intestinal and gastric tumor numbers, respectively, compared with Apc1638N animals. The intestinal tumors of both groups showed no significant differences in grading and staging. Loss of heterozygosity analysis at the Apc and E-cadherin loci in both intestinal and gastric Apc+/1638N/E-cad+/− tumors revealed loss of the wild-type Apc allele in most cases, whereas the wild-type E-cadherin allele was always retained. This was supported by a positive, although reduced, staining for E-cadherin of intestinal tumor sections.

Conclusions: Introduction of the E-cadherin mutation in Apc1638N animals enhances Apc-driven tumor initiation without clearly affecting tumor progression.

Malignant tumors are the result of accumulation of several genetic alterations. Several lines of evidence indicate that mutations of the adenomatous polyposis coli (APC) gene represent one of the earliest genetic events in intestinal tumor development. First, germline APC mutations are responsible for familial adenomatous polyposis, a dominant condition characterized by multiple colorectal adenomatous polyps in the colon and rectum.1,2 Germline mutations in the mouse Apc gene also result in a dominant predisposition to the development of multiple tumors in the intestine and elsewhere.3–6 In accordance with Knudson’s 2-hit hypothesis, somatic mutations at the wild-type APC allele can be detected in most intestinal tumors of both human and murine origin.5,7–10 Lastly, the majority of sporadic colorectal tumors carry APC mutations with similar frequencies between early and more advanced tumor stages.11

The APC gene encodes a 312-kilodalton protein shown to participate in several cellular processes, including cell cycle regulation, apoptosis, cell adhesion, cell migration, microtubule assembly, and signal transduction.12 We showed recently that the tumor-suppressing function of APC resides in its capacity to properly regulate intracellular β-catenin levels, whereas its COOH-terminal domains seem to be less relevant for its function in development and tumorigenesis.13 β-Catenin functions as a signaling molecule within the Wnt signal transduction pathway by associating in the nucleus with members of the lymphoid enhancer factor/T-cell factor family of transcriptional activators, thereby modulating the transcription of target genes.14 In the absence of a Wnt-signal, APC promotes down-regulation of β-catenin, thereby preventing its signaling activity.15 As a result of APC mutations, β-catenin constitutively transduces signals to the nucleus. Accordingly, oncogenic β-catenin mutations that make it resistant to proteolytic degradation are found in sporadic colorectal tumors without APC mutations.16

In addition to its role in the Wnt-signaling pathway, β-catenin binds to the cytoplasmic domain of E-cadherin, thus mediating cell-to-cell interactions by linking it to the actin cytoskeleton.17–20 Whereas loss of APC

Abbreviations used in this paper: APC, adenomatous polyposis coli; LOH, loss of heterozygosity.
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function seems to be involved in initiation of intestinal tumor development, inactivation of E-cadherin is generally associated with later stages of tumor progression because disruption of E-cadherin function correlates with increased tumor invasiveness.\textsuperscript{21–23} Therefore, by reducing the expression of E-cadherin in animals with a targeted Apc mutation, one may expect an increased invasive potential of the developing intestinal tumors. However, because β-catenin appears to exist in a state of dynamic equilibrium with E-cadherin and Apc,\textsuperscript{24,25} reducing E-cadherin levels may also potentially affect intestinal tumor initiation by increasing the amount of intracellular β-catenin available for signaling. Reduction of E-cadherin levels may also contribute to tumorigenesis by affecting its cell-adhesive and associated functions.\textsuperscript{26–29} A possible role for E-cadherin in tumor initiation is supported by the presence of germline mutations in families predisposed to gastric cancer development\textsuperscript{30–32} and mutations in early-stage gastric and breast cancers.\textsuperscript{33–35}

To address the issue of “cross talk” between cell-to-cell adhesion and signal transduction, we have bred the Apc\textsuperscript{1638N} model with mice carrying a targeted E-cadherin mutation.\textsuperscript{36} The double heterozygous Apc\textsuperscript{1638N}/E-cad\textsuperscript{+/−} mice show a marked increase in tumor number along the upper gastrointestinal tract without any clear modification of tumor invasiveness or malignant potential. These results indicate that haploinsufficiency at the E-cadherin locus is synergistic with mutation of the Apc gene in gastrointestinal tumor initiation.

**Materials and Methods**

**Animals**

C57BL/6Jic0(B6)-Apc\textsuperscript{+1638N} females were mated with E-cadherin\textsuperscript{+/-} males of mixed Ola129/129J genetic background to obtain Apc\textsuperscript{+1638N}/E-cad\textsuperscript{+/-} and Apc\textsuperscript{+1638N}/E-cad\textsuperscript{+/-} mice on an F1(B6xOla129/129J) background. The same cross resulted in Apc\textsuperscript{+/+}/E-cad\textsuperscript{+/-} and Apc\textsuperscript{+/+}/E-cad\textsuperscript{+/-} littermates as controls. The experiments were approved by the animal experimental commission (UDEC) of the Medical Faculty of the Leiden University (Leiden, The Netherlands; permission 9613).

**Gross Study and Tissue Processing**

All animals were killed between 6 and 9 months of age, after which the entire intestine was removed and opened longitudinally. Sections of approximately 10 cm were spread out flat on filter paper, fixed overnight at 4°C in Notox (Earth Safe Industries, Inc., Bellemead, NJ), and transferred to 70% ethanol. The size and location of macroscopically visible tumors were determined, followed by resection and embedding of lesions in paraffin according to standard procedures. In addition, a number of freshly isolated tumors were snap-frozen in Tissue-tek OCT compound (Sakura B.V., Zoeterwoude, The Netherlands). Cutaneous cysts and desmoids were counted as described previously.\textsuperscript{6} Liver and lungs of all animals were investigated for the presence of metastases by macroscopic and microscopic analysis. Tumor numbers were statistically evaluated by the Mann–Whitney exact test.

**Histopathologic Classification of Gastrointestinal Tumors**

Tumors were classified according to standard criteria,\textsuperscript{37} considering a given tumor malignant only if invasion (lamina propria or beyond) was present. The invasion was defined by the presence of stromal fibroblastic reaction (desmoplasia). Tumors were graded according to the extension of glandular differentiation,\textsuperscript{38} and staged according to the depth of invasion in the intestinal wall and the presence of lymph node or systemic metastases. Both lungs and liver were embedded in toto, and serial H&E-stained sections were screened for the presence of microscopic metastases by 2 independent observers (S.D.-C. and A.L.).

**Tumor DNA Isolation**

Tumor DNA isolation from microdissected paraffin or cryosections was performed essentially as previously described.\textsuperscript{10} Briefly, six 16-μm-thick sections were placed on glass slides, deparaffinized, rehydrated, and briefly stained with hematoxylin. The area containing the tumor cells was subsequently microdissected under a dissection microscope using as reference an H&E-stained 5-μm section. The isolated neoplastic tissue was transferred in 250-μL extraction buffer (10 mmol/L Tris-Cl [pH 8.0], 100 mmol/L NaCl, 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and 300 μg/mL proteinase K) and incubated at 55°C for 40 hours. A second aliquot of 60 μg of proteinase K was added after 20 hours of incubation. Cellular proteins were removed by a phenol/chloroform and chloroform extraction followed by a 30-minute precipitation at −20°C with 125 μL 7.5 mol/L Na2Ac, 20 μg glycogen, and 625 μL ethanol. The precipitate was resuspended in 50 μL TE 4 (10 mmol/L Tris-Cl [pH 8.0] and 0.1 mmol/L EDTA).

**Loss of Heterozygosity Analysis at the Apc and E-cadherin Locus**

Loss of the wild-type Apc and E-cadherin allele was analyzed by amplifying in a single reaction both the wild-type and targeted alleles.\textsuperscript{10} The following primers were used for E-cadherin: 1533 (5′-CCTCTCTTTTGGACAGGAACCTCGT-3′) and 1534 (5′-CAAGGCCAGGAGTGACGACACTGATG-3′), resulting in a 190-base pair (bp) product diagnostic of the wild-type allele, and E-cadneo71 (5′-GCAAAACCACACTGCGAC-3′) that in combination with 1533 results in a 198-bp product diagnostic of the targeted allele. For the Apc locus, the following primers were used: Apc-A2 (5′-TCAGCCCAGGCAAAAGCTCA-3′) and
Immunohistochemical staining of E-cadherin in paraffin sections was performed with a monoclonal antibody (clone 34) raised against the COOH-terminal 150 residues of E-cadherin (Transduction Laboratories, Lexington, KY). Sections were rehydrated, blocked in PBS containing 5% nonfat dry milk, and subsequently incubated for 16 hours at 4°C with the primary antibody diluted 1:100 in PBS/5% nonfat dry milk. After 3 washes in PBS containing 0.2% Tween 20, the sections were incubated for 1 hour in a 1:250 dilution of a horseradish peroxidase–conjugated F(ab′)2, fragment goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). The peroxidase reaction was developed with 0.01% hydrogen peroxide in 0.05% dianaminobenzidine tetrahydrochloride in 50 mmol/L Tris-HCl (pH 7.6).

**Results**

**Tumor Size and Numbers in the Gastrointestinal Tract of Apc+/−/1638N/E-cad+/− Mice**

To study the interaction between cell-to-cell adhesion and signal transduction in the gastrointestinal epithelium, we have bred Apc1638N mice4,6 with mice carrying a targeted E-cadherin mutation.36 The gastrointestinal tracts of the compound Apc+/−/1638N/E-cad+/− mice were analyzed for the presence of tumors and compared with their Apc+/−/1638N, E-cad+/−, and wild-type littermates. All animals were killed between 6 and 9 months of age (mean age for all groups, 7.5 months). As expected, no tumors were observed in wild-type (n = 10) and E-cad+/− (n = 9) animals, whereas Apc+/−/1638N mice (n = 17) had an average of 1.24 tumors per animal in the small intestine. A dramatic effect on tumor multiplicity along the gastrointestinal tract was observed in the compound heterozygous Apc+/−/1638N/E-cad+/− animals (Table 1). The number of small intestinal tumors in these mice (n = 18) showed a significant 9-fold increase (average, 11.3 tumors per animal) compared with the Apc+/−/1638N group (P < 0.0001, Mann–Whitney test). A similar increase was observed for gastric tumors. Only 4 of 11 Apc+/−/1638N mice had a single gastric tumor, but all 14 investigated Apc+/−/1638N/E-cad+/− animals had developed at least 1 tumor. The average number of macroscopically detectable gastric lesions was increased by more than

### Table 1. Gastrointestinal Tumor Numbers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Intestine</th>
<th>Stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. studied</td>
<td>No. of tumors</td>
</tr>
<tr>
<td>+/+</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>E-cad+/−</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Apc+/−/1638N</td>
<td>17</td>
<td>1.24 ± 1.1</td>
</tr>
<tr>
<td>Apc+/−/1638N/E-cad+/−</td>
<td>18</td>
<td>11.3 ± 5.1</td>
</tr>
</tbody>
</table>
Figure 1. Histogram showing the size distribution of intestinal tumors in Apc+/1638N and Apc+/1638N/E-cad+/− animals.

5-fold, from 0.36 to 2.0 tumors per animal (P < 0.001, Mann–Whitney test). Cutaneous cysts and desmoid lesions, characteristic of the Apc1638N mutation, were not affected by the E-cadherin mutation (data not shown).

In contrast with the increase in tumor numbers, tumor size was not clearly affected by the E-cadherin haploinsufficiency. The Apc+/1638N tumors (n = 21) had grown to an average diameter of 2.32 ± 0.83 mm, which is similar to the 2.01 ± 0.83 mm observed in 124 tumors derived from 11 Apc+/1638N/E-cad+/− animals. As shown in Figure 1, also the distribution in tumor size between both groups is comparable, whereby the tumors of the Apc+/1638N/E-cad+/− animals even show a slight tendency toward smaller tumors. Hence, these results are indicative of an effect of E-cadherin haploinsufficiency on gastrointestinal tumor multiplicity in the Apc1638N model, rather than on the rate at which these tumors grow.

Morphology and Malignant Potential of the Gastrointestinal Tumors

Detailed histologic analysis of 15 Apc+/1638N small intestinal tumors showed evidence of stromal invasion in all cases. The same was observed in 117 of 124 Apc+/1638N/E-cad+/− tumors. Most of the lesions found in both genotypes can therefore be regarded as carcinomas. Submucosal invasion was observed in 10 of 15 Apc+/1638N tumors and 62 of 124 Apc+/1638N/E-cad+/− tumors. No diffuse growth pattern characteristic for a loss of adhesive function was observed in any of the tumors. Serial sectioning of the liver and lungs could not detect any metastases, neither in the Apc+/1638N group nor in the group with additional E-cadherin mutation. Although we cannot exclude the presence of micrometastases, the absence of any identifiable metastasis in the double heterozygous animals with the 9-fold higher tumor burden suggests that this is an uncommon event. Therefore, both the degree of local invasion and the metastatic potential of the Apc1638N tumors are not affected by the E-cadherin mutation.

All gastric tumors were located in the fundus and pyloric region. The lesions of both groups showed variable proportions of gland-like structures, indicating an intestinal phenotype without any evidence of diffuse tumor growth. Examples of gastric lesions are shown in Figure 2.

E-cadherin Is Expressed in Tumors of Both Genotypes

We have previously shown that in the majority of Apc1638N intestinal tumors, the wild-type copy of Apc is somatically lost.10,41,42 To assess whether loss of the wild-type E-cadherin allele underlies tumor formation in double heterozygous Apc+/1638N/E-cad+/− animals, 16 intestinal and 16 gastric tumors were investigated by LOH analysis. Twelve of 16 intestinal tumors tested showed loss of the wild-type Apc allele. Gastric tumors showed loss of the wild-type Apc allele in 9 of 16 cases with borderline results in 2 other tumors. In contrast, all gastric and intestinal tumors showed retention of the wild-type E-cadherin allele (Figure 3).

Immunohistochemical analysis was performed on cryosections of tumors from both genotypes with the rat monoclonal antibody DECMA, directed against residues 407–434 of E-cadherin.39,40 Expression could be detected in all tumors, although the staining intensity was clearly reduced compared with the normal intestinal mucosa (Figure 4). The staining pattern was similar for tumors of both genotypes with no obvious quantitative differences. This was also the case for a rabbit polyclonal β-catenin antibody (Figure 4).

Both the LOH and immunostaining results with the DECMA antibody suggest that wild-type E-cadherin is still expressed in Apc+/1638N/E-cad+/− tumors. However, it is plausible that a truncating mutation in the second half of the protein disrupts E-cadherin function. Therefore, 10 paraffin-embedded Apc+/1638N/E-cad+/− intestinal tumors were stained with a monoclonal antibody raised against the COOH-terminal 150 residues of E-cadherin. All tumors had retained immunoreactivity, strongly suggesting that wild-type E-cadherin is still present in the majority of tumors (data not shown).

Discussion

Complete loss of E-cadherin function is generally associated with the acquisition of an invasive phenotype.21–23 However, inactivation has also been observed in early-stage gastric and lobular breast cancers characterized by a diffuse growth pattern.33–35 We now show
that heterozygosity for an inactivating $E$-cadherin mutation in $Apc^{1638N}$ animals results in a strong increase of the number of gastrointestinal tumors without clearly affecting malignant potential. Interestingly, no LOH at the $E$-cadherin locus was observed in the corresponding tumors, and $E$-cadherin expression was still detectable by immunohistochemistry. No diffuse growth pattern was observed in any of the tumors, as is frequently the result of a complete inactivation of $E$-cadherin function.$^{43–45}$ Thus, introduction of the $E$-cadherin mutation in $Apc^{1638N}$ animals mainly seems to affect tumor formation rather than tumor progression.

A number of not mutually exclusive mechanisms can be envisaged to explain this increase in tumor multiplicity. First, $E$-cadherin haploinsufficiency could enhance $Apc$-driven tumorigenesis by increasing the pool of cytoplasmic $\beta$-catenin available for Wnt signaling. Defects in the Wnt signaling pathway underlie intestinal tumor formation, as indicated by the presence of mutations in either $APC$ or $\beta$-catenin in most colorectal cancers.$^{7,16}$ We have recently shown that (1) the tumor-suppressing activity of $Apc$ lies in its ability to prevent $\beta$-catenin to signal through the Wnt pathway,$^{13}$ and (2) that $Apc$ mutations are selected upon their capacity to inactivate the $\beta$-catenin down-regulating activity in intestinal tumorigenesis in the mouse.$^{46}$ Tumor formation is most efficient if all $Apc$ domains involved in $\beta$-catenin regulation are removed. In other words, once both copies of $Apc$ become mutated, a stronger $\beta$-catenin/Wnt signal to

Figure 2. Example of a gastric lesion observed in an $Apc^{+}/1638N/E$-cad$^{+/−}$ animal. (A) Normal gastric epithelium from the pyloric region of the stomach. (B) The upper part shows an invasive intestinal-type adenocarcinoma. Gastric mucosa epithelium shows interspersed goblet cells as sign of intestinal metaplasia (arrowhead) and regenerative gastric epithelium. (C) A detail of an invasive adenocarcinoma revealing pleomorphic cells with prominent nucleoli and tubular formation (arrowhead). The irregular glands in the center represent early invasion of the stroma (arrow).

Figure 3. Example of tumor LOH analysis at the $Apc$ and $E$-cadherin locus. C1–C2, DNA isolated from normal intestinal mucosa; T1–T5, DNA isolated from tumor samples. All tumors show a clear loss of the wild-type $Apc$ allele, but all have retained the wild-type $E$-cadherin allele.
the nucleus increases the chance of intestinal tumor formation.

Although β-catenin has distinct roles in cell adhesion and signaling,47–49 several investigations have shown that cadherins may act as modulators of the Wnt signaling pathway. Increasing the level of cadherin in Xenopus embryos depleted the cytoplasmic pool of β-catenin and interfered with Wnt signal-mediated axis formation.47,50 Similar studies in Drosophila showed that overexpression of cadherin mimicked the wingless phenotype observed when Armadillo, the Drosophila homologue of β-catenin, is mutated. This defect could be rescued by increasing Armadillo levels.48 In colorectal cancer cells, expression of cadherin derivatives inhibited β-catenin–mediated transactivation of a Wnt reporter.51,52 The opposite effect, i.e., increasing the signaling pool of β-catenin by reducing cadherin levels, has also been shown.53 Haploinsufficiency for cadherin in Drosophila diminished the wingless defect of an intermediate mutant of Armadillo, presumably by releasing some of the normally membrane-bound Armadillo and making it available for signaling. Thus, a considerable amount of experimental evidence supports a substantial role for cadherins in modulating Wnt signal-related processes. Accordingly, the reduced amount of E-cadherin in Apc1638N/E-cad1/2 animals could result in an enlarged intracellular pool of β-catenin, thus enhancing Wnt signaling and the likelihood of tumor formation to occur. The increased signaling activity most likely becomes apparent only once Apc is temporarily inactivated by an extracellular Wnt signal or when both copies of Apc become mutated. That Apc inactivation still appears to be a rate-limiting step for tumor development is supported by the loss of the wild-type Apc allele in 9–11 of 16 gastric and 12 of 16 intestinal Apc1638N/E-cad1/2 tumors, similar to LOH frequencies previously observed in Apc1638N tumors on various genetic backgrounds.10,41,42 Moreover, heterozygosity at the E-cadherin locus itself is not sufficient to trigger tumorigenesis, as confirmed by the lack of tumors in E-cad+/− mice. However, no clear increase in β-catenin levels was observed in the Apc+/1638N/E-cad+/− tumors in comparison with Apc+/1638N tumors, although it should be noted that the immunofluorescence method used is not appropriate for quantitative comparisons. Further experimentation is needed to show that the reduced E-cadherin levels affect Wnt signaling.

E-cadherin participates in a complex network of interactions between adhesion receptors, cytoskeletal proteins, and several signaling pathways.26–29 Therefore, changes at adherens complexes may result in alterations of cell shape and polarity, proliferation, cell motility, attachment to the extracellular matrix, and cytoskeletal organization. This was also shown by the introduction of a dominant negative N-cadherin transgene in the murine intestinal tract.54 Expression of the transgene interfered with normal cadherin function, resulting in a reduced
epithelial barrier function, altered proliferation, migration, and apoptosis along the crypt-villus axis, leading to inflammatory bowel disease and development of adenomas. In the normal intestinal mucosa of Apc<sup>+1638N</sup>/E-cad<sup>+/−</sup> animals, no such abnormalities were observed, suggesting that sufficient E-cadherin function is available to support its normal functions. Nevertheless, we cannot exclude that an undetected change in cell-to-cell adhesion and subsequent changes in cellular functions may be responsible for the observed increase in tumor numbers.

The 5-fold increase of gastric tumors in Apc<sup>+1638N</sup>/E-cad<sup>+/−</sup> mice is interesting in view of the recent observation suggesting that germline mutations at the human E-cadherin gene are responsible for familial diffuse gastric cancer. Somatic E-cadherin mutations have also been observed at high frequency in sporadic gastric cancers of the diffuse type and in invasive, lobular breast carcinomas. In most cases, these mutations are accompanied by a loss of the second allele. Thus, as a consequence of the complete loss of E-cadherin function, there seems to be a morphologic shift to a diffuse growth pattern. No mutations have been detected in intestinal-type gastric lesions and colorectal cancers. Nevertheless, a high proportion of colorectal and some intestinal-type gastric cancers show reduced expression of E-cadherin. The latter has been explained by a number of mechanisms, including transcriptional down-regulation either by promoter methylation or binding of transcriptional repressors such as Snail, which are upregulated in tumor cells. This resembles the situation of the gastrointestinal tumors of the Apc<sup>+1638N</sup>/E-cad<sup>+/−</sup> animals, in which no diffuse growth pattern has been observed while retaining E-cadherin immunoreactivity at reduced levels, which suggests that inactivation of only one E-cadherin allele contributes to tumor development.

At 6–9 months of age, many Apc<sup>+1638N</sup>/E-cad<sup>+/−</sup> animals showed severe signs of morbidity as a result of the high tumor multiplicity. This precluded analysis of the tumor phenotype at later time points. However, at the time of analysis, no obvious differences in histopathologic appearance were observed between the tumors of both genotypes, as also confirmed by the similar tumor size and degree of invasion. No E-cadherin loss was observed among Apc<sup>+1638N</sup>/E-cad<sup>+/−</sup> tumors, and E-cadherin expression in the tumors was still detectable by immunohistochemistry. Therefore, the level of E-cadherin in Apc<sup>+1638N</sup>/E-cad<sup>+/−</sup> mice seems to suffice to prevent further progression toward a more invasive tumor phenotype compared with the Apc<sup>+1638N</sup> controls. However, besides a complete inactivation of E-cadherin, the acquirement of a metastatic phenotype requires the functional dysregulation of additional proteins.

In conclusion, introduction of the E-cadherin mutation in Apc<sup>+1638N</sup> animals affects tumor initiation in the gastrointestinal tract rather than progression toward malignancy. This is most likely a result of the overlapping functions of E-cadherin in controlling both Wnt signal transduction and cell adhesion.

References


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Activating point mutations in cyclin-dependent kinase 4 are not seen in sporadic pituitary adenomas, insulinomas or Leydig cell tumours

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(V V Vax and R Bibi contributed equally to this work)

Abstract

Cell cycle dysregulation is one of the defining features of cancer. Cyclin-dependent kinase 4 (CDK4), together with its regulatory subunit cyclin D, governs cell cycle progression through the G1 phase. Cyclin-dependent kinase inhibitors, including p16INK4A (encoded by CDKN2A), in turn regulate CDK4. In particular, dysregulation of the p16/CDK4/cyclin D complex has been established in a variety of types of human tumours. Dominant activating mutations affecting codon 24 of the CDK4 gene (replacement of Arg24 by Cys or His) render CDK4 insensitive to p16INK4A inhibition and are responsible for melanoma susceptibility in some kindreds. However, ‘knock-in’ mice homozygous for the CDK4R24C mutation were noted to develop multiple neoplasia, most commonly including endocrine tumours: pituitary adenomas, benign or malignant insulinomas and Leydig cell tumours. We therefore speculated that sporadic human endocrine tumours might also harbour such mutations. The aim of the current study was to analyze the CDK4 gene for the two characterized activating mutations, R24C and R24H, in sporadic human pituitary adenomas, insulinomas and Leydig cell tumours. We used DNA extracted from 61 pituitary adenomas, and paired tumorous and neighboring normal genomic DNA extracted from 14 insulinoma and 6 Leydig cell tumour samples. Genomic DNA from patients with familial melanoma harbouring the R24C or the R24H mutations served as positive controls. All samples were subjected to PCR, mutation-specific restriction digests and/or sequencing. Both methodologies failed to detect mutations at these two sites in any of the sporadic endocrine tumours including pituitary adenomas, benign or malignant insulinomas or Leydig cell tumours, while the positive controls showed the expected heterozygote patterns. Protein expression of CDK4 was demonstrated by immunohistochemistry and Western blotting in pituitary and pancreatic samples. These data suggest that the changes in the regulatory ‘hot-spot’ on the CDK4 gene, causing various endocrine tumours including pituitary adenomas, benign or malignant insulinomas or Leydig cell tumours, while the positive controls showed the expected heterozygote patterns. Protein expression of CDK4 was demonstrated by immunohistochemistry and Western blotting in pituitary and pancreatic samples. These data suggest that the changes in the regulatory ‘hot-spot’ on the CDK4 gene, causing various endocrine tumours in CDK4R24C/R24C mice, are not a major factor in sporadic pituitary, insulin β-cell or Leydig cell tumorigenesis.

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Introduction

Abnormalities in cell cycle stimulators and inhibitors are increasingly recognized as crucial factors in tumorigenesis. Examples of cell cycle stimulators include the cyclins and cyclin-dependent kinases (CDKs), whilst examples of inhibitors of the cell cycle include the retinoblastoma protein (Rb) and the CDK inhibitors (INK4 and Cip/Kip group; Fig. 1) (Pavelitch 1999). In G1 phase, various mitogenic signals activate the cell cycle via the cyclin D/CDK4 complex which, in turn, can be inhibited by the CDK inhibitor p16INK4 (p16, encoded by CDKN2A) via binding to CDK4. Mutation in the CDK4 gene was first described in melanoma cells (Wölfel et al. 1995): the mutation involved an arginine to cysteine change (Arg→Cys) at codon 24 (R24C) as a result of a single nucleotide change (CGT to R24H). This mutation is an activating mutation, as it results in growth advantages
because it prevents p16 binding, while maintaining the ability of CDK4 to form a functional kinase with cyclin D. It was previously known that ~20% (range <5% to >50% in individual studies) of familial melanoma kindreds harbour a mutation in p16, but germline CDK4 mutations have also been identified in three familial melanoma cases (Kefford et al. 2002). Zuo et al. (1996) examined 31 melanoma families with no p16 mutations, two of which harboured a heterozygote R24C mutation. A second germline mutation was described by Soufir et al. (1998) in one of 48 French melanoma families. The striking point about this second mutation is that it occurs in the same Arg24 codon as the previously mentioned R24C mutation, directly involved in binding to p16 proteins, but in this case arginine is exchanged for histidine (Arg→His) as a consequence of a single nucleotide change (CGT→CAT). To test its in vivo relevance, the CDK4 R24C allele was ‘knocked in’ to the germline of mice, using homologous recombination (Rane et al. 1999, Sotillo et al. 2001). Whilst expression of this mutant gene did not cause any gross developmental defects, the mice were noticed to be on average 5–20% larger than their wild-type controls. Spontaneous melanoma was not observed in these mice, but following treatment with two carcinogenic compounds, 7,12-dimethylbenz[a]anthracene (DMBA) and 12-0-tetradecanoylphorbol-13-acetate (TPA), they developed large papillomas that frequently progressed to invasive skin carcinomas. Interestingly, CDK4 R24C mice developed a wide spectrum of tumours with a 55% incidence of endocrine neoplasia, specifically 3 types of endocrine tumours: those of Leydig cell origin were the most frequent (62% prevalence), followed by pancreatic β-islet cells (35%) and pituitary cells (22%). Pituitary tumours expressed a variety of hormones and were of adenohypophysyal origin (80%). This is in contrast to pRb, p27 or p18INK4c knockout mice: these animals also

Figure 1  Cell cycle stimulators and inhibitors. Rb is a tumour suppressor protein which controls the G1-S transition partly by blocking the activity of a group of transcription factors, E2F (Zhang et al. 1999, Malumbres & Barbacid 2001). Progressive phosphorylation of Rb caused by the various cyclin/CDK complexes results in the release of the E2F complex, therefore enabling DNA synthesis to occur. The CDK inhibitor, p16, acts specifically by binding and inhibiting the cyclin D/CDK4 complex.
commonly develop pituitary tumours, which mostly originated from the adrenocorticotropic hormone (ACTH)-secreting pars intermedia.

CDK4 knockout animals have also been generated (CDK4<sup>−/−</sup>) (Rane et al. 1999). Both male and female mice lacking the CDK4 protein were 50% smaller than wild-type animals, and were sterile, with reduced numbers of Leydig cells and abnormal corpus luteum formation. CDK4<sup>−/−</sup> mice also showed features of diabetes mellitus: histology of the pancreatic tissue revealed severe deformity and a reduction in the size of the islet cells, principally the β islet cells. These results indicate that CDK4 is required for postnatal proliferation of β islet cells, as both the CDK4<sup>+/-K24C</sup> and CDK4<sup>−/−K24C</sup> mice develop β cell tumours while the CDK4<sup>−/−</sup> knockout types were born with normal islet cell number and morphology but develop diabetes soon after.

Based on the above findings, we investigated the possible presence of these two activating mutations of CDK4 in sporadic human pituitary adenomas, insulinomas and Leydig cell tumours.

Materials and Methods

Tumour specimens

Pituitary samples Human pituitary adenomas were obtained at the time of transphenoidal surgery and were stored at −80 °C. All studies were approved by the local Ethics Committee. The tumour type was determined on the basis of clinical and biochemical findings before surgery, and histological and immunocytochemical analysis of the removed tissue sample. Tumour extension was determined directly at surgery as well as by pre-operative magnetic resonance imaging (MRI). We investigated 61 pituitary tumours (46 cDNA and 15 genomic DNA samples). Of these, 17 were classified as active somatotroph adenomas (10 somatotroph, 5 somatomammotroph and 2 sparsely-granulated somatotroph), 36 were clinically non-functioning pituitary adenomas (cNFPA, 29 null cell adenomas, 2 silent corticotrophs, 4 silent gonadotrophs and 1 oncocytoma), 5 were corticotroph adenomas, 3 were active lactotroph adenomas; 35 of the pituitary tumours (57.4%) had invasive extension. The clinicopathological details of the patients are included in Table 1. For the immunoblotting studies autopsy samples were used as normal pituitaries.

Insulinoma samples Genomic DNA from 14 insulinomas was extracted from formalin-fixed, paraffin-embedded tissue. Eight of these patients had isolated tumours (one of them was an islet cell carcinoma), whilst 5 of the patients had insulinomas as part of multiple endocrine neoplasia type 1 (MEN1) syndrome. One of the samples analysed was that of a metastatic lesion from the liver of a patient with an insulinoma. In all cases, both tumorous tissue and normal tissue surrounding the tumour were analysed. The data of the patients with insulinomas are included in Table 2.

Leydig cell tumour samples Six cases of Leydig cell tumours were investigated. In all cases, both tumorous tissue and normal testis tissue surrounding the tumour were analysed from microdissected samples. The data of patients with Leydig cell tumours are included in Table 3.

Nucleic acid extraction Total RNA prepared from fresh-frozen pituitary tumours was transcribed to cDNA as previously described (Korbonits et al. 2001). Genomic DNA from tissue of pituitary adenomas was extracted using the QIAamp DNA Mini Kit (Qiagen). Microdissected sections from formalin-fixed, paraffin-embedded samples were taken from 10-μm unstained sections of insulinomas, Leydig cell tumours and histologically normal pancreatic and testis tissue. The DNA extraction was performed as follows (Diaz–Cano & Brady 1997): 10 ml volume lysis buffer was made, consisting of 200 μl proteinase K (200 μg/ml), 500 μl Tris pH 8·3 (50 mM), 50 μl carrier glycerol (100 μg/ml), 100 μl Tween 20 (0·5%), 150 μl calcium chloride (1·5 mM) and finally 9000 μl sterile water. A portion (300 μl) of this lysis buffer was added to each microcentrifuge tube with the tissue fragments. The samples were kept at 61 °C for three days while an additional 6 μl proteinase K were added to each tube every 12 h. At the end of the third day, one drop of Chelex beads and, after 5 min, 2 μl 0·5 M EDTA were added to each tube. Samples were precipitated with ice-cold absolute ethanol in the presence of 0·3 M sodium acetate, pH 5·2. All samples were then dissolved in 15 μl TE buffer, pH 8·0, and stored in the −20 °C freezer until further use.

Positive controls were genomic DNA (gDNA) samples extracted from blood from patients with familial melanoma syndrome due to CDK activating mutations (Zuo et al. 2001). Genomic DNA from tissue of pituitary adenomas was extracted using the QIAamp DNA Mini Kit (Qiagen). Microdissected sections from formalin-fixed, paraffin-embedded samples were taken from 10-μm unstained sections of insulinomas, Leydig cell tumours and histologically normal pancreatic and testis tissue. The DNA extraction was performed as follows (Diaz–Cano & Brady 1997): 10 ml volume lysis buffer was made, consisting of 200 μl proteinase K (200 μg/ml), 500 μl Tris pH 8·3 (50 mM), 50 μl carrier glycerol (100 μg/ml), 100 μl Tween 20 (0·5%), 150 μl calcium chloride (1·5 mM) and finally 9000 μl sterile water. A portion (300 μl) of this lysis buffer was added to each microcentrifuge tube with the tissue fragments. The samples were kept at 61 °C for three days while an additional 6 μl proteinase K were added to each tube every 12 h. At the end of the third day, one drop of Chelex beads and, after 5 min, 2 μl 0·5 M EDTA were added to each tube. Samples were precipitated with ice-cold absolute ethanol in the presence of 0·3 M sodium acetate, pH 5·2. All samples were then dissolved in 15 μl TE buffer, pH 8·0, and stored in the −20 °C freezer until further use.

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### Table 1 Characteristics of patients with pituitary tumours

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Clinical diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Tumour size</th>
<th>Immunohistochemistry</th>
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<td>GH, weakly for PRL</td>
</tr>
<tr>
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<td>Macro</td>
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<td>Macro</td>
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<td>Negative</td>
</tr>
<tr>
<td>50</td>
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<tr>
<td>53</td>
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<td>M</td>
<td>Macro+SSE</td>
<td>FSH, weakly for LH</td>
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</tr>
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<td>ACTH</td>
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<td>59</td>
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<td>33</td>
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<td>PRL</td>
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<tr>
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<td>Prolactinoma</td>
<td>63</td>
<td>F</td>
<td>Macro+SSE</td>
<td>PRL</td>
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</table>

NA, not available; CNFPA, clinically non-functioning pituitary adenoma; macro, macroadenoma; micro, microadenoma; SSE, suprasellar extension; CS, cavernous sinus extension; GH, growth hormone; PRL, prolactin; αSU, α-subunit; FSH, follicle-stimulating hormone; LH, luteinising hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone.
(250 ng RNA equivalent), 2·5 µl Thermophilic DNA Poly 10 × buffer (Promega), 0·25 µl 20 mM dNTP mix, 0·5 µl of each primer at 0·4 µM, 0·25 µl 25 mM MgCl₂, and 0·125 µl Taq DNA polymerase (5 U/µl). For the pituitary samples 35 cycles were performed at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, after a first denaturing cycle at 95°C for 5 min. A final extension cycle of 10 min at 72°C was used. For gDNA extracted from formalin-fixed, paraffin-embedded tissue, a 25 µl PCR reaction was used with 2 µl gDNA, 2·5 µl 10 × Qiagen PCR buffer (Qiagen), 0·5 µl 20 mM dNTP mix, 0·5 µl of each primer at 0·4 µmol/l, 0·25 µl MgCl₂ (25 mM), 0·25 µl Hotstar Taq DNA Polymerase and 5 µl Q-Solution. Forty PCR cycles were performed at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, after a first denaturing cycle at 95°C for 15 min. A final extension cycle of 10 min at 72°C was used. To confirm successful amplification the PCR products were run on 2% ethidium bromide-stained agarose gels and ΦX174 DNA/HinfI markers (Promega).

### Table 2 Characteristics of patients with insulinomas

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA</td>
<td>F</td>
<td>Insulinoma</td>
<td>Insulin</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>M</td>
<td>Islet cell tumour</td>
<td>Insulin</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>F</td>
<td>Insulinoma, MEN1</td>
<td>Insulin, VIP, PPP, glucagon</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>F</td>
<td>Insulinoma</td>
<td>Insulin</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>F</td>
<td>Insulinoma</td>
<td>Insulin</td>
</tr>
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<td>F</td>
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<td>Insulin</td>
</tr>
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<td>7</td>
<td>53</td>
<td>M</td>
<td>Insulinoma, MEN1</td>
<td>Insulin</td>
</tr>
<tr>
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<td>51</td>
<td>M</td>
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<tr>
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<td>Metastatic islet cell tumour (liver sample)</td>
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<tr>
<td>10</td>
<td>42</td>
<td>M</td>
<td>Islet cell carcinoma</td>
<td>Insulin, occasional glucagon</td>
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<td>Insulinoma</td>
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<td>Insulin</td>
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<td>36</td>
<td>F</td>
<td>Insulinoma MEN1</td>
<td>Insulin, glucagon, PPP</td>
</tr>
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<td>M</td>
<td>Insulinoma malignant met</td>
<td>Insulin negative</td>
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</tbody>
</table>

NA, not available; MEN1, multiple endocrine neoplasia type 1; VIP, vasoactive intestinal peptide; PPP, pancreatic polypeptide; SS, somatostatin; met, metastasis.

### Table 3 Characteristics of patients with Leydig cell tumours

<table>
<thead>
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<th>Patient no.</th>
<th>Age</th>
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<th>Histology</th>
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<tbody>
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<tr>
<td>3</td>
<td>36</td>
<td>M</td>
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<tr>
<td>4</td>
<td>36</td>
<td>M</td>
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<tr>
<td>5</td>
<td>77</td>
<td>M</td>
<td>Benign Leydig cell tumour, no invasion</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>M</td>
<td>Benign Leydig cell tumour, no invasion</td>
</tr>
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</table>

NA, not available.

### Restriction enzyme reactions and sequencing

The Leydig cell tumours and 15 of the pituitary adenoma samples were studied using restriction enzyme analysis while a representative group of insulinomas was studied by both sequencing and restriction enzyme analysis. The two single nucleotide substitutions within codon 24 create recognition sites for two restriction endonucleases. The Arg→His mutation (CAT) creates a site for Hsp92II (Promega), while the Arg→Cys mutation (TGT) creates a recognition site for StuI (Promega). The PCR primers were designed so that the products would only be cut once by the restriction enzymes in the presence of a mutation. Ten microlitres of the PCR product were digested with 0·5 µl of the Hsp92II or StuI enzyme, 2 µl Restriction Enzyme 10 × buffer K, 0·2 µl acetylated bovine serum albumin and 7·3 µl water to bring the total volume to 20 µl. The reactions were mixed gently by pipetting and incubated at 37°C for 2 h and 65°C for 25 min at the end of the digestion period. Ten microlitres of the digest were then used to run on a 4·5% metasieve agarose gel (Flowgen, Ashby, Leicestershire, UK) together with uncut PCR samples. Direct sequencing was performed on all the pituitary cDNA samples and all the insulinomas using the ABI Prism 3700 DNA Analyzer - for details see Kola et al. (2003).

### Immunohistochemistry

The sections were mounted on positively-charged microscope slides (Superfrost Plus; Fisher Scientific, Fair Lawn, NJ, USA) and baked at 60°C for 2 h. The slides were routinely dewaxed and rehydrated. The endogenous
peroxidase activity was then quenched with 0·5% H₂O₂ in methanol for 10 min. A microwave antigen retrieval method (20 min in 10 mM citrate buffer, pH 6·0, at 600 watts) was used, followed by incubation with polyclonal horse serum (20 min, 1:100 dilution: Dako, Glostrup, Denmark) and with rabbit polyclonal anti-CDK4 antibody (clone sc-260, overnight, 4°C), at 2 µg/ml (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then sections were serially incubated with biotinylated antimouse antibody (30 min, 1:200 dilution; Dako), and peroxidase-labelled avidin–biotin complex (60 min, 1:100 dilution; Dako). All incubations were performed in a moist chamber at room temperature. The reaction was developed under microscopic control, using 3,3′-diaminobenzidine tetrahydrochloride with 0·3% H₂O₂ as chromogen (Sigma Chemical Co., St Louis, MO, USA), and the sections were counterstained with haematoxylin. Both positive (reactive lymph node) and negative (omitting the primary antibody) controls were simultaneously run. The specificity of the reaction was tested using blocking peptide in competition studies (sc-260-P, Santa Cruz Biotechnology, Inc.).

Western blotting

Pituitary tissue samples were homogenised in Cytobuster protein extraction reagent (Novagen, CN Biosciences, Nottingham, Notts, UK). The protein concentration of the lysates was normalized using the Bradford assay (Bio-Rad, Hemel Hempstead, Middlesex, UK). Samples (10 µg) were subjected to 10% SDS-PAGE separation, with protein transfer to PVDF nitrocellulose membrane. The membrane was blocked with 5% non-fat milk for 90 min and incubated overnight at 4°C using rabbit polyclonal anti-CDK4 antibody (1:1000, clone sc-260, Santa Cruz Biotechnology, Inc.) as the primary antibody. The membrane was washed 3 times with PBS containing 0·05% Tween and subsequently incubated with horse-radish peroxidase (HRP)–conjugated anti-rabbit antibody as the secondary antibody (1:10 000, Dako) for 2 h at room temperature. A chemiluminescent peroxidase substrate, ECL Plus (Amersham–Pharmacia, Amersham, Bucks, UK), was applied according to the manufacturer’s instructions, and the membranes were exposed briefly to X-ray film. Equal protein loading was determined by stripping the PVDF membrane with Tris hydrochloride, SDS and 2-mercaptoethanol, and re-probing for β actin (1:5000, ab6276, Abcam, Cambridge, Cambs, UK).

Results

The area of CDK4 gene containing codon 24 was successfully amplified in all the samples using the gDNA or the cDNA primers. The positive control samples showed the expected heterozygote pattern by both restriction enzyme analysis (Fig. 2) and sequencing (Fig. 3). No abnormal restriction enzyme patterns or sequencing results were detected at the area including codon 24 of the CDK4 gene in any of the 61 pituitary adenomas, the 14 insulinoma samples or in the 6 Leydig cell tumours (Figs 2 and 3). CDK protein expression was detected in insulinomas with immunohistochemistry, where the specificity of the staining was shown by blocking the staining with the antigen (Fig. 4). Western blotting on normal pituitary and pituitary adenomas showed variable expression of the expected 34 kDa size protein, with no consistent difference from normal tissue (Fig. 5).

Discussion

We have studied the possible involvement of 2 different activating point mutations in the cell cycle regulator CDK4 in sporadic endocrine tumours, based on findings in the corresponding transgenic mouse model which develops insulinomas, Leydig cell tumours and pituitary adenomas when such mutations are present (Sotillo et al. 2001). No mutations were found in any of these neoplasms at codon R24, or in the surrounding area.
CDK4 in sporadic endocrine tumours

from codon 1 to codon 41. However, CDK4’s role cannot be totally ruled out as several non-contiguous amino-acid sequences on CDK4 are required for binding to p16 (codons 22, 24, 25, 97 and 281) (Ceha et al. 1998) and only codons 22, 24 and 25 were investigated by sequencing in our study, although no germline or sporadic CDK4 mutations outside codons 22–24 have been reported to date. We were able to detect CDK4 protein in both the insulinoma and pituitary samples using immunohistochemistry and immunoblotting, and significantly altered expression of CDK4 protein could not be detected.

Loss of regulation of the G1 checkpoint appears to be an extremely common event among virtually all types of tumours. A number of genetic and epigenetic mechanisms have been shown to be responsible for this process. The two types of proteins that are of central importance are the cyclins and cyclin-dependent kinases. CDK4 and CDK6, which associate with the D-type cyclins, are expressed in most tissues but the abundance of the two proteins may be cell specific. Over-expression of cyclin D1, due to amplification of the cyclin D1 gene, has been reported in many tissues and cell lines including the breast, oesophagus and colorectal tissue. It was therefore reasonable to speculate that its regulatory counterpart, CDK4, could harbour genetic alterations that may lead to tumorigenesis.

Considering that CDK4 is a potential oncogene, mechanisms of activation could include gene amplification, over-expression and decreased degradation, other than activating mutations. In humans, point mutations in the CDK4 gene have been described in familial melanoma. The CDK4 gene is amplified and over-expressed in a number of human tumours including the gliomas, sarcomas, breast tumours and colorectal carcinomas: 6 of 67 osteosarcoma samples demonstrated CDK4 gene amplification using quantitative Southern blot analysis (Wei et al. 1999). In colorectal carcinomas immunohistochemical analysis using antibodies to CDK4 revealed a positive correlation between elevated levels of CDK4 and enhanced dysplasia of the adenomas (Bartkova et al. 2001). This study again supported the notion that defects in CDK4 could lead to disruption of cell cycle control. Further support for the proliferative properties of CDK4 comes from transgenic mice over-expressing the CDK4 gene under a keratin 5 promoter (Miliani de Marval et al. 2001).

![Figure 3 Sequencing traces from a pituitary adenoma (no mutation) and the two positive control samples (heterozygote R24C and R24H mutation).](image)
The mice developed severe dermal fibrosis, epidermal hyperplasia and hypertrophy, independent of D-type cyclin expression. Furthermore, expression of CDK4 has been shown to have prognostic value: cyclin D1 and CDK4 are possible candidates for prognostic markers in laryngeal squamous cell carcinomas (Dong et al. 2001). Over-expression of CDK4 was observed in 47% (48/102) of laryngeal tumours and was significantly associated with overall poor survival. Both cyclin D1 and CDK4 over-expression were independent prognostic factors, but the poorest outcome was associated with co-overexpression of both cyclin D1 and CDK4. Pituitary adenomas have been studied for abnormalities in the p16 pathway, including CDK4 for the Arg→Cys mutations at codon 24, but no mutation was found (Simpson et al. 2001). The latter study examined 45 human pituitary tumours and looked at several components of the cell cycle i.e. Rb, p16, cyclin D1 and CDK4, to assess their overall contribution to pituitary tumorigenesis. Whilst a significant percentage of the tumours had abnormal expression of Rb, p16 and cyclin D1 proteins, not a single pituitary sample showed the CDK4 R24C mutation. Ten of the samples were randomly selected to undergo sequencing of the codon 24 region, but no mutations were detected.
However, the majority of the samples were not tested for the Arg→His mutation. In a recent paper it has been suggested that in CDK4R24C/R24C transgenic mice the activating mutation does not just facilitate tumorigenesis but also aids escape from cellular senescence and contact-induced growth arrest (Rane et al. 2002).

While p16 changes are common in melanoma families, CDK4 changes are rare (Goldstein et al. 2002). A 13-fold increase in the incidence of pancreatic (exocrine) cancer in melanoma-prone kindreds with p16 mutations has been described (Goldstein et al. 1995), and the risk of breast cancer is also increased (Borg et al. 2000). No report has been published on endocrine abnormalities in these families harbouring p16 or CDK4 abnormalities. Interestingly, a recent preliminary report describes a patient with deletion of chromosome 9p (encompassing the p16-CDKN2A locus): this patient at the age of 16 developed a number of melanomas and dysplastic naevi as well as a gonadotrophin-secreting pituitary tumour (Kumar et al. 2002).

In conclusion, the importance of the CDK4/p16 pathway has been established in a range of different tumour types, suggesting that the development of cyclin-dependent kinase inhibitor drugs could lead to useful anti-neoplastic agents (Sausville 2002). CDK4 R24C plays a major role in mouse endocrine tumorigenesis, but in the current study we could not detect any CDK4 abnormalities in sporadic endocrine human tumours. This suggests that CDK4 may play a different role in the human as compared with mouse endocrine cells.

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COMMENTARY

Hashimoto’s thyroiditis shares features with early papillary thyroid carcinoma

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Hashimoto’s thyroiditis shares features with early papillary thyroid carcinoma

Neoplastic transformation is a multistep process that results in a continuous spectrum from the normal (physiological) state to a fully established neoplasm. The gold standard for diagnosis of papillary thyroid carcinoma is conventional histology, the essential element being the characteristic nuclear features, regardless of whether papillary structures are present or not. However, other criteria are being used increasingly in the diagnosis of neoplasms, including immunohistochemical staining and molecular profile. The RET/PTC gene rearrangement is highly specific for papillary thyroid carcinoma and is associated with the characteristic nuclear features seen in papillary thyroid carcinoma. There is an overlap in the morphological features, immunohistochemical staining pattern, and most importantly, molecular profile between papillary thyroid carcinoma and Hashimoto’s thyroiditis. Although considered a ‘benign’ condition, Hashimoto’s thyroiditis almost always harboura a genetic rearrangement that is strongly associated with and is highly specific for papillary thyroid carcinoma. Submicroscopic foci of papillary thyroid carcinoma must be present in Hashimoto’s thyroiditis, although the clinical behaviour is still benign. Further studies are required to predict which foci will progress to papillary thyroid carcinoma.

Keywords: papillary thyroid carcinoma, Hashimoto’s thyroiditis, RET/PTC rearrangement, diagnostic criteria, molecular diagnosis

Introduction

Neoplastic transformation is a multistep process that results in a continuous spectrum from the normal (physiological) state to a fully established neoplasm. This biological concept contrasts with the dualistic point of view in which a lesion is either benign or malignant with no intermediate stage. Some tumours do not have a benign histological counterpart, but it does not mean that a non-metastasizing condition sharing genetic and kinetic features does not exist. A recent commentary in Histopathology examined the diagnostic criteria used to make a diagnosis of papillary thyroid carcinoma, and the author concluded that there is no benign counterpart to this tumour based on morphological features.1 However, upon reviewing the evidence regarding the molecular basis of papillary thyroid carcinoma, alternative hypotheses emerge (Figure 1). The definition of neoplasms is still descriptive and mainly based on histological features. However, depending on the diagnostic criteria, the definition of papillary thyroid carcinoma can be expanded.

Current diagnostic criteria for papillary thyroid carcinoma

Assuming that a follicular differentiation has been demonstrated, the diagnosis of papillary thyroid carcinoma requires consideration of papillary formations,
nuclear features, and a supportive immunohistochemical profile. The most recent Armed Forces Institute of Pathology fascicle defines papillary thyroid carcinoma as ‘A malignant epithelial tumour showing evidence of follicular cell differentiation and characterized by the formation of papillae and/or a set of distinctive nuclear features’. This definition allows the diagnosis of papillary thyroid carcinoma to be made in the absence of characteristic nuclear features. In fact, nuclear features are the essential diagnostic component, and although frequently associated with papillae, the diagnosis of papillary thyroid carcinoma can be made in their absence. Because of the close correlation of the nuclear features with the genetic pattern of papillary thyroid carcinoma, they will be considered together in a later section.

Malignancy refers to tumour capability of distant growth, which is mainly predicted by the presence of tissue infiltration. In papillary thyroid carcinoma, tissue infiltration can be a prognostic indicator: diffuse sclerosing papillary thyroid carcinoma has a higher incidence of lymph node and systemic metastases, and tumours without identifiable infiltrating growth have lower rates of metastases. Improvements in diagnostic tools have resulted in an increased incidence of early papillary thyroid carcinoma with very low metastatic potential. These lesions still fulfill the papillary thyroid carcinoma morphological criteria and are therefore considered malignant. However, microscopic papillary thyroid carcinoma and encapsulated papillary thyroid carcinoma would represent low-grade papillary neoplasms with clinically benign behaviour.

Papillary structures not fulfilling papillary thyroid carcinoma criteria have been described in other neoplastic and non-neoplastic lesions such as follicular adenoma, multinodular goitre and Graves’ disease. In 21 cases of encapsulated thyroid nodules with papillary structures and nuclear features falling short of the diagnostic criteria for papillary thyroid carcinoma, lymph node metastases were not present. Focal, limited, nuclear features of papillary thyroid carcinoma were seen in nine cases. Nuclear diameter was less than that seen in papillary thyroid carcinoma. Variable immunoreactivity with markers CK19 and HBME-1 and negative immunoreactivity for Ret in all but one case further support the authors’ conclusion that such cases do not represent papillary thyroid carcinoma. The papillary structures in these cases stained positively with the proliferation marker MIB1, indicating that these structures are unlikely to represent degenerative changes.

Although conventional histology is the cornerstone of papillary thyroid carcinoma diagnosis, immunohistochemical techniques can be a useful adjunct. Immunexpression of highmolecular weight cytokeratins, S100 protein and HBME-1 helps to identify papillary thyroid carcinoma. Papillary thyroid carcinomas stain positively for S100 protein but benign papillary hyperplastic lesions do not. HBME-1 positivity indicates malignancy but not papillary differentiation. In addition, certain low molecular weight cytokeratins, particularly cytokeratin 19 (CK19), but also CK17 and CK20, are positive in papillary thyroid carcinoma and the intensity of staining is greater in areas with obvious nuclear features. Focal CK19 staining may be found in benign lesions, but diffuse positivity is characteristic of papillary thyroid carcinoma. An antibody panel composed of HBME-1, CK19 and Ret may be useful in difficult diagnostic cases. Immunostaining with vascular endothelial growth factor may be helpful as a prognostic marker, as the number of positive follicular cells and staining intensity are associated with metastatic papillary thyroid carcinoma and a significantly shorter recurrence-free survival compared with non-metastatic papillary thyroid carcinoma.

Molecular diagnostic criteria for papillary thyroid carcinoma: the \textit{RET/PTC} rearrangement

The crux of papillary thyroid carcinoma diagnosis relies on nuclear changes: overlapping, elongated ground glass nuclei with grooves and pseudoinclusions are characteristic and are the most reliable features. The demonstration of \textit{RET/PTC} expression by immunohistochemistry has been highly associated with an irregular nuclear contour and a euchromatic appearance compared with non-expressing cells. A Ras independent signalling pathway downstream of Ret/ptc leads to restructuring of the nuclear envelope and chromatin. The gold standard nuclear features for the diagnosis of papillary thyroid carcinoma are related to the \textit{RET/PTC} rearrangement. Therefore, the diagnostic core of papillary thyroid carcinoma at the molecular level should be this rearrangement, in order...
to provide reproducibility and correlation with standard diagnostic procedures. This approach has been successfully applied in the subclassification of Hurthle cell tumours, identifying the Hurthle cell variant of papillary thyroid carcinoma.\textsuperscript{12}

The \textit{RET}/\textit{PTC} oncogene, a rearranged form of the proto-oncogene \textit{RET}, is highly specific for papillary thyroid carcinoma.\textsuperscript{13,14} Its detection may therefore be useful as a diagnostic aid. Located on chromosome 10, \textit{RET} is thought to have a role in the control of neural crest migration and neuroectodermal differentiation during embryonic development. It is not normally expressed in normal follicular epithelium. Increased expression of \textit{RET} is found in tumours derived from cells of neural crest origin, e.g. neuroblastomas, phaeochromocytomas, medullary thyroid carcinomas.

Rearrangements of \textit{RET} lead to gene expression and occur in almost 50\% of papillary thyroid carcinomas. They consist of fusion of the tyrosine kinase encoding domain with the 5’ domain of ubiquitously expressed genes, which provide the promoter and dimerization functions, necessary for the constitutive activation of Ret/ptc. Oncogenic activation of \textit{RET} is apparently due to ligand-independent constitutive ectopic tyrosine kinase activity. The most commonly detected rearrangement (\textasciitilde70\%) is \textit{RET}/\textit{PTC1}, in which the tyrosine kinase domain is fused to the 5’ end of the \textit{H4} gene, also located on chromosome 10. Inversion of the long arm of chromosome 10 provides the structural basis for this. Activation of \textit{RET}/\textit{PTC} has been detected in a large proportion of occult microscopic papillary thyroid carcinomas and it is thought therefore that it represents an early event in the process of follicular cell transformation.\textsuperscript{15,16}

The prevalence of \textit{RET} rearrangements is higher in radiation-induced papillary thyroid carcinomas (16/19 (84\%) cases versus 3/20 (15\%) controls). \textit{RET}/\textit{PTC1} being the most frequent.\textsuperscript{17} This is in contrast to the distribution of \textit{RET}/\textit{PTC} rearrangements in papillary thyroid carcinomas from children exposed to high doses of radioactive iodine isotopes after the Chernobyl accident, in whom \textit{RET}/\textit{PTC3} is predominant.\textsuperscript{18} Novel \textit{RET} rearrangements have been detected in this population, designated \textit{RET}/\textit{PTC5}, 6, 7 and 8.\textsuperscript{19} Also, the presence of \textit{RET} rearrangement has been related to aggressive behaviour: rearrangement-positive papillary thyroid carcinomas were in a more advanced pT category and more frequently in the pN1 category at presentation than rearrangement-negative papillary thyroid carcinomas. \textit{RET}/\textit{PTC3} was related to the solid variant of papillary thyroid carcinoma, \textit{RET}/\textit{PTC1} more frequently to typical papillary structures. Post-Chernobyl papillary thyroid carcinomas therefore demonstrate a characteristic distribution of gene rearrangements that lead to typical phenotypes.

\textit{RET}/\textit{PTC} rearrangements are present in early stages of tumorigenesis (including the smallest morphological counterpart or microscopic papillary thyroid carcinoma)\textsuperscript{15} as demonstrated by immunohistochemical expression and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses.\textsuperscript{16} Multiple coexistent \textit{RET}/\textit{PTC} rearrangements are the rule for multicentric papillary thyroid carcinoma, suggesting that each tumour has a different clonal origin.\textsuperscript{16,20} These findings are not only of academic interest. They contribute to better staging of patients and may provide prognostic information. Multifocal monoclonal papillary thyroid carcinoma shows intraglandular dissemination with increased cellular migration capacities and should be considered as a more advanced stage tumour than multicentric oligoclonal papillary thyroid carcinoma, for which each tumour should be independently staged.

Conflicting results are reported regarding the prognostic role of \textit{RET}/\textit{PTC} activation.\textsuperscript{21} In a recent study, \textit{RET}/\textit{PTC} rearrangements were detected using a real-time RT-PCR assay in seven of eight anaplastic thyroid carcinomas.\textsuperscript{22} It is recognized that anaplastic thyroid carcinomas derive from papillary thyroid carcinoma, and it has been suggested therefore that \textit{RET}/\textit{PTC} expression could be a marker of tumour virulence in papillary thyroid carcinoma. Conversely, Ret was detected in 64.6\% of 127 papillary thyroid carcinomas by immunohistochemistry using a polyclonal antibody against its tyrosine kinase domain and no correlation with patient outcome was found after a mean follow-up period of 18 years.\textsuperscript{23}

Other genetic changes have been reported in both sporadic and familial papillary thyroid carcinoma. The genetic background of familial papillary thyroid carcinoma is, however, poorly understood and certainly a role for the \textit{RET}/\textit{PTC} rearrangement has not been found yet. Lesueur \textit{et al.} studied cases of familial non-medullary thyroid cancer in 56 families. Of the 138 tumours examined, there were 113 papillary thyroid carcinomas. Linkage to \textit{RET}, \textit{MNG1} and \textit{TCO} was excluded, reinforcing the hypothesis that this is a genetically heterogeneous condition.\textsuperscript{24} Other genetic syndromes with which papillary thyroid carcinoma has been associated include familial adenomatous polyposis and papillary renal neoplasia.\textsuperscript{25} Regarding sporadic papillary thyroid carcinoma, several other activated oncogenes have been identified in thyroid malignancies, including \textit{RAS}, the mutated form of the \textit{α} subunit of the Gs-protein (GSP) and \textit{TRK}, but they are not specific to papillary thyroid carcinoma and are probably associated with the later stages of tumorigenesis.\textsuperscript{26}
A high prevalence of TP53 point mutations has been found in anaplastic thyroid carcinomas, but not in differentiated follicular tumours. Therefore, these genes are less useful for the molecular diagnosis of papillary thyroid carcinoma.

**The RET/PTC rearrangement in other conditions**

Although we cannot readily identify a precursor lesion for papillary thyroid carcinoma on the basis of morphological features, our knowledge of the molecular genetic basis of papillary thyroid carcinoma may shed some light on the issue. Patients with Hashimoto’s thyroiditis have a higher incidence of thyroid cancer and most of these cases are papillary thyroid carcinomas. The nuclear features and genetic rearrangements of papillary thyroid carcinoma can be seen in Hashimoto's thyroiditis. The two conditions share the same immunohistochemical profile.

Examination of thyroid resection specimens from 21 patients with Hashimoto’s thyroiditis revealed that six had concomitant papillary thyroid carcinoma. A RT-PCR assay was used to detect mRNA expression for RET/PTC1 and RET/PTC3. In all but one patient expression of one or both oncogenes was detected. In 71% of patients expression of both RET/PTC1 and RET/PTC3 was detected. Of note, all of the patients with Hashimoto’s thyroiditis but without papillary thyroid carcinoma expressed RET/PTC1 and/or RET/PTC3. In six cases of lymphocytic thyroiditis-associated papillary thyroid carcinoma from two unrelated families, the same RET/PTC rearrangement was detected in cases from each family: RET/PTC1 in one family and RET/PTC3 in the other. The authors were unable, however, to demonstrate a germ-line mutation and interestingly, the rearrangement was detected in adjacent non-tumoral thyroid tissue. Perhaps these cases are comparable therefore to those of Hashimoto’s thyroiditis-associated papillary thyroid carcinoma.

Di Pasquale et al. examined 33 cases of Hashimoto’s thyroiditis with concomitant thyroid carcinoma, 30 of which were papillary thyroid carcinomas. Frequent features of these papillary thyroid carcinomas were prominent stromal desmoplasia and a pseudovascular growth pattern. Four cases showed ‘atypical nodules’, two of which were associated with papillary thyroid carcinoma. The nodules were solid, cellular lesions with no follicular or papillary architecture and demonstrated some but not all of the nuclear features of papillary thyroid carcinoma. Two of the four nodules showed positive CK19 staining. Three of the four nodules showed positive immunohistochemical staining for RET/PTC. RET/PTC rearrangements are present in microscopic papillary thyroid carcinoma and Hashimoto’s thyroiditis, providing a molecular link for these conditions; additional genetic changes, however, must contribute to the expansion of these early lesions, resulting in tumour progression. Further studies are required to identify useful molecular predictors of progression.

In conclusion, papillary thyroid carcinoma and Hashimoto’s thyroiditis overlap in morphological features, immunohistochemical pattern, and most importantly, molecular profile. Although considered a ‘benign’ condition, Hashimoto’s thyroiditis can harbour the RET/PTC rearrangement which is an early, specific marker that is strongly associated with papillary thyroid carcinoma. Is Hashimoto’s thyroiditis a submicroscopic counterpart of papillary thyroid carcinoma? The atypical nodules described by Di Pasquale et al. may also be a morphological expression of progression to a tumour nodule demonstrating full features of papillary thyroid carcinoma. It has been established that papillary thyroid carcinoma is more frequent in Hashimoto’s thyroiditis and also that the incidence of occult microscopic papillary thyroid carcinoma is high. This raises management issues for patients with Hashimoto’s thyroiditis. Can we predict which patients will develop potentially metastasizing papillary thyroid carcinoma? To answer this question, further studies are needed to establish the predictive value of the RET/PTC rearrangement and other markers in patients with Hashimoto’s thyroiditis, microscopic papillary thyroid carcinoma and encapsulated papillary thyroid carcinoma.

**References**


Abstract The cell kinetic of prostatic intraepithelial neoplasia (PIN) is poorly understood. Herein we report the kinetic pattern of PIN, both not associated (primary) and associated (secondary) with coexistent invasive carcinoma (PCa). Surgical specimens collected in 20 cases of primary PIN, 20 of secondary PIN and 20 of PCa were studied by MIB-1 immunostaining, in situ end-labeling (ISEL) and DNA histogram analysis, and the cell density in each case was estimated using the formula $N = (n/p)^2$. Fifty high-power fields (HPF), or the complete lesion if smaller, were screened in each lesion, and both mean and standard deviation were recorded. Statistical differences were studied by means of Fisher’s exact test. ISEL indices were significantly ($P<0.0001$) lower in PCa (0.1±0.3) than in primary PIN (0.5±0.3), while the MIB-1 indices were similar in both conditions ($P=0.56$). Statistically significant differences were also detected for both MIB-1 and ISEL indices when secondary PIN (MIB-1 1.9±0.7, ISEL 3.7±3.3) was compared with primary PIN (MIB-1 2.5±2.1, ISEL 0.5±0.3) and PCa ($P<0.0001$). In terms of cellularity, primary PIN (26.3±7.1) revealed scores significantly lower ($P<0.0001$) than those recorded in PCa (39.0±8.8) and secondary PIN (32.9±14.3). In conclusion, early prostatic tumor is mainly defined by down-regulated apoptosis rather than by increased proliferation. Secondary PIN displays unique kinetic features suggesting an evolved stage of primary PIN.

Key words Prostatic intraepithelial neoplasm · Precancerous lesion · Intraductal extension · Cell kinetics · DNA-ploidy

Introduction

Currently, high-grade prostatic intraepithelial neoplasia (PIN) is the most likely precursor of invasive prostatic carcinoma (PCa) [2, 3] and has been reported both as an isolated finding (primary PIN) and coexisting with invasive adenocarcinomas (secondary PIN) [4, 22].

The normal cellular turnover is maintained by a strict balance between proliferation and apoptosis [27, 43], studied by several techniques including immunohistochemistry, in situ end-labeling (ISEL) of fragmented DNA, and DNA-ploidy analysis. Controversial results have been reported on cell kinetics in PIN because of the heterogeneity of cases (especially regarding total androgen ablation) [32, 34, 35] and the diversity of techniques and quantification methods for both proliferating cells [23, 33, 45] and apoptotic cells [18, 31–34, 47]. Additionally, no attempts to differentiate primary from secondary PIN have been published. Therefore, the biological significance of both types of PIN remains unknown, and their potential implications for therapeutic approaches need to be determined.

The purpose of this study was to characterize the kinetic features of both primary and secondary PIN in a series of surgical specimens. Combined quantitative analyses of proliferation and apoptosis markers on tissue sections were performed. Ultimately, those parameters were to help define the kinetic of tumor progression in PCa.
Materials and methods

Case selection

Forty consecutive patients were included in this study, 20 with PIN only (in a background of nodular hyperplasia) and 20 with co-existent low-grade PCa and PIN in the same specimen. All patients showed enlarged prostate and high serologic levels of prostate-specific antigen (PSA); either radical prostatectomy (all patients with PCa and 16 without PCA) or transurethral resection (4 patients without PCa) was undertaken as appropriate treatment. None of these patients underwent androgen deprivation therapy before the surgical resection, and the treatment was selected according to clinicopathological criteria, such as PSA levels or imaging findings. No histological evidence of extraprostatic tumor extension was detected, and no patient with primary PIN developed pathologically proven PCa during the study (mean follow-up of 92 months).

The surgical specimens were routinely processed and representative samples were taken (following the standard criteria of sampling in prostatic resections) to establish the histological diagnosis [24]. Bostwick’s criteria were used to classify high-grade PIN [4]. Appropriate archival material was available for additional studies in [24]. Bostwick’s criteria were used to classify high-grade PIN [4].

MIB-1 immunostaining

The sections were mounted on positively charged microscope slides (Superfrost Plus, Menzel, Germany) and baked at 60°C for 2 h. After routine dewaxing (xylene), rehydration (ethanol), and endogenous peroxidase quenching (0.5% H2O2 in methanol, for 2 h. After routine dewaxing (xylene), rehydration (ethanol), and endogenous peroxidase quenching (0.5% H2O2 in methanol), the sections were microwaved in 10 mM citrate buffer, pH 6.0, for 20 min and serially incubated with polyclonal horse serum (1/100 dilution, Dako Denmark) for 20 min, specific primary antibody (4 µg/ml, Dianova Hamburg, Germany) overnight at 4°C, biotinylated anti-mouse antibody (1/200 dilution, Dako Denmark) for 30 min, and peroxidase-labeled avidin–biotin complex (1/100 dilution, Dako Denmark) for 60 min. All incubations were performed in a moist chamber at room temperature unless otherwise specified. The reaction was developed under microscopic control, using 3,3’-diaminobenzidine tetrahydrochloride as chromogen (Sigma, St. Louis, Mo.), and the sections were counterstained with hematoxylin.

Both positive (reactive lymph node) and negative (omitting the primary antibody) controls were run simultaneously.

ISEL of DNA fragments

Since extensive DNA fragmentation is an important characteristic of apoptosis, visualization of DNA breaks has proved useful in the identification of apoptotic cells [43]. This extensive DNA fragmentation results in a high density of 5’-protruding ends, which can be detected using the Klenow fragment of DNA polymerase I with a mixture of labeled nucleotides [48]. Briefly, the sections were deparaffinized and hydrated as described above. After incubation in 2×SSC buffer (80°C, 20 min) and protein digestion (500 µg/ml pronase in 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5% SDS, at room temperature for 25 min), the sections were deparaffinized and hydrated as described above. After incubation in 2×SSC buffer (80°C, 20 min) and protein digestion (500 µg/ml pronase in 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5% SDS, at room temperature for 25 min), the sections were deparaffinized and hydrated as described above. After incubation in 10 mM Mg-CI, 1 mM dithiothreitol, 250 µg/ml BSA with 100 µM of each dNTP, maintaining a proportion of 11-digoxigenin-dUTP/ATTP of 0.35/0.65; 2 h at 37°C). The dig-labeled DNA fragments were immunoenzymatically detected using an antidigoxigenin polyclonal Fab fragment labeled with alkaline phosphatase (1/100 dilution, Boehringer-Mannheim, Germany); the enzymatic reaction was developed under microscopic control with nitroblue-tetrazolium and X-phosphate [12]. The sections were counterstained with diluted hematoxylin (25%), dehydrated, and mounted.

Both positive (reactive lymph node) and negative (omitting DNA polymerase in the enzymatic incubation) controls were simultaneously run.

Quantification of positive nuclei

At least 50 HPF (50 HPF=7.6 mm2) were screened, or the complete lesion if smaller (18 patients, 12 without PCa and 6 with PCa), in each pathologic group. Both the number of positive nuclei per HPF and the number of neoplastic ductal cells intercepted by the microscope field diameter were recorded. The last score was used to estimate the number of neoplastic cells per HPF using the formula N=n(πr2)/2, where N is the number of estimated cells per HPF and n, the number of cells intercepted by the microscope field diameter [11, 25, 44]. The number of positive nuclei was always expressed per HPF and per 1,000 neoplastic cells. Both the average and the standard deviation (SD) values were calculated as representative scores per pathologic condition and patient.

The threshold of positivity was experimentally established at the positive control in each staining batch. Only those nuclei showing staining features similar to those of their corresponding positive control were considered positive for a given marker (MIB-1, ISEL). We only kept, for quantification, those nuclei in the same focus level taken as representative of any given HPF.

Slide cytometric analysis of DNA content

Feulgen-stained sections were used for DNA quantification [1], using the Cell Analysis System (CAS) model 200 and Quantitative DNA Analysis software (Becton-Dickinson). At least 200 nuclei were evaluated from each focus and the results recorded separately. From the same slide, both lymphocytes and nonneoplastic ductal cells were used as diploid controls. External diploid controls (rat hepatocytes provided by Becton-Dickinson) were included in each staining batch to normalize results. Several 5-µm sections were used for this analysis, according to previously published protocols that have proven valid in such material [19, 29, 46].

Only complete, nonoverlapping and focused nuclei were interactively selected, beginning in the most cellular area. The histogram of nuclear optical density was used to evaluate the DNA index (as referred to their corresponding diploid controls), the proliferation rate (PR = S + G, + M / G, + S + G, + M, expressed as a percentage), and the ratio between the nuclear area and the DNA content of the cells in each cell cycle phase. The last variable was also referred to the corresponding values in the nonneoplastic ductal cells of the prostate to normalize the results.

Statistical analysis

The average and SD of every variable in each pathologic condition (primary PIN, secondary PIN, and PCa) were statistically compared by means of Fisher’s exact tests. The data were stratified in two subsets, above and below the corresponding threshold, in each pathologic group. The thresholds were 3% for MIB-1 immunorexpression, 0.4% for ISEL of DNA fragments, and 35 cells/HPF for cellularity estimation. Differences were considered statistically significant if P<0.05.

Results

Patients ages varied between 51 and 64 years (average 56) in the group without PCa and between 60 and
Combined analysis of kinetic indices in each pathologic condition showed a direct correlation between proliferation and apoptosis in primary PIN, while there was an inverse correlation between the two in both low-grade PCs and secondary PIN (Fig. 1). These opposite patterns were mainly due to very low apoptosis rates in low-grade PCs (in 80% of these cases ISEL indices were lower than 0.4%) and both relatively low PR and comparatively high ISEL in secondary PIN (Table 1, Figs. 1, 2). MIB-1 indices below 3% were observed in 86% of cases but 42% of primary PIN revealed the highest score for invasive low-grade PCs (Fig. 1), which was characterized by low proliferation (MIB-1 index ≤3% in 80% of cases) and high apoptosis (ISEL index ≥0.4% in 80% of cases).

The statistical analysis revealed no significant differences between primary PIN and low-grade PCs for either MIB-1 index (P=0.56) or ISEL index (P=0.09). Although a discrete increase in the MIB-1 index was observed in low-grade PCs (Table 1), the cases were equally distributed around (50% below and 50% above) 3% (Fig. 1). Both primary PIN and low-grade PCs showed low ISEL indices, which were lower in low-grade PCs than in primary PIN but with wide intergroup overlapping, precluding any statistical conclusion (Table 1).

Anyway, the progression primary PIN → low-grade PCs was kinetically related to down-regulation of apoptosis: 80% of cases of low-grade PCs showed an ISEL index ≤0.4%, while only 50% of primary PIN revealed similar scores (Fig. 1). That tendency can only be confirmed in groups with less variability and with more cases included in the analysis.

On the other hand, secondary PIN showed statistically significant kinetic features from both primary PIN and low-grade PCs (P<0.0001 in all comparisons, for both MIB-1 and ISEL indices). Secondary PIN was kinetically characterized by the lowest MIB-1 index and the highest ISEL index (Table 1), the reverse pattern of low-grade PCs (Fig. 1), which was characterized by low proliferation (MIB-1 index ≥3% in 86% of cases) and high apoptosis (ISEL index >0.4% in 80% of cases). The slide cytometric analysis of DNA content revealed at least two G0/G1 cells in all cases but 4 (2 primary PIN and 2 invasive low-grade PCs). The average DNA content of the prevalent G0/G1 peak showed a progressive increase from primary PIN through invasive low-grade PCs to secondary PIN (Table 2), with diploid G0/G1 cells predominating in primary PIN and low-grade PCs and hyperdiploid G0/G1 cells in secondary PIN (Fig. 4).

The presence of multiple cases with two G0/G1 peaks precluded an appropriate and reliable evaluation of proliferative index from the DNA histograms in each group.

Low-grade PCs displayed the highest cellularity scores, whereas primary PIN had the lowest scores and secondary PIN revealed average values between those for primary PIN and low-grade PCs, but with the highest variability (Table 1). No differences were detected in a comparison between low-grade PCs and secondary PIN. Low-grade PCs showed slightly higher cellularity than secondary PIN, but the high variability of the latter resulted in a

### Table 1
Proliferative and apoptotic features in intraductal and invasive neoplasms of the prostate (PIN prostatic intraepithelial neoplasia, PCA prostatic carcinoma)

<table>
<thead>
<tr>
<th></th>
<th>Primary PIN (Mean ± SD)</th>
<th>Invasive PCA (Mean ± SD)</th>
<th>Secondary PIN (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIB-1 index</td>
<td>2.5±2.1</td>
<td>3.5±1.7</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>ISEL index</td>
<td>0.5±0.3</td>
<td>0.1±0.3</td>
<td>3.7±3.3</td>
</tr>
<tr>
<td>Cellularity</td>
<td>26.3±7.1</td>
<td>39.0±8.8</td>
<td>32.9±14.3</td>
</tr>
</tbody>
</table>

71 years (average 68) in the group with PCs. All cases showed tufting or micropapillary high-grade PIN with no evidence of comedonecrosis, regardless of whether or not there was any association with PCs. The PCAs were scored (Gleason) ≤4 in all patients. The MIB-1 index showed a progressive increase from secondary PIN through primary PIN to low-grade PC, while the ISEL index revealed the lowest scores in low-grade PC and the highest in secondary PIN (Table 1).

### Table 2
Slide cytometric analysis of DNA content in intraductal and invasive neoplasms of the prostate

<table>
<thead>
<tr>
<th></th>
<th>Primary PIN (Mean ± SD)</th>
<th>Invasive PCA (Mean ± SD)</th>
<th>Secondary PIN (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear area</td>
<td>42.80±6.34</td>
<td>41.11±4.25</td>
<td></td>
</tr>
<tr>
<td>(NuA)</td>
<td>33.04±5.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA index (DI)</td>
<td>1.11±0.16</td>
<td>1.12±0.11</td>
<td>1.22±0.07</td>
</tr>
<tr>
<td>NuA / DI</td>
<td>29.74±5.10</td>
<td>38.28±5.66</td>
<td>33.62±3.48</td>
</tr>
</tbody>
</table>

![Fig. 1](Image) Kinetic profiles in prostatic intraepithelial neoplasia (PIN) and low-grade prostatic carcinoma (PCA). Each bar represents the percentage of cases revealing a given feature (proliferation or apoptosis). The cases were categorized in two groups for both proliferation index (MIB-1, threshold 3%) and apoptotic index (in situ end-labeling [ISEL], threshold 4%). The progression from primary PIN to low-grade PC was kinetically defined by significant down-regulation of apoptosis with a moderate but not significant increase in proliferation. The opposite pattern characterized the transition primary PIN into secondary PIN. Both proliferation and apoptosis were significantly different in secondary PIN (Fisher’s exact test).
broad undefined window precluding any statistical conclusion. Even after cellularity categorization, no differences were detected: 50% of low-grade PCa and 60% of secondary PIN revealed scores of ≤35 cells/HPF ($P=0.1004$). However, primary PIN was proven statistically different from both low-grade PCa and secondary PIN ($P<0.0001$ in both cases). Primary PIN showed the lowest cellularity of all groups (Table 1), 80% of them had ≤35 cells/HPF.

Discussion

Two main inferences could be drawn in this study. First, PIN is kinetically different according as whether there is coexistent PCa. Secondly, apoptosis plays an essential role in early steps of prostate tumorigenesis, while proliferation seems to be of secondary importance in this progression. We discuss these points below.

Different cell kinetic profiles characterized PIN associated and not associated with coexistent PCa. The relatively low proliferation and relatively high apoptosis of PIN with coexistent PCa (secondary PIN) were demonstrably statistically different from those of PIN with no PCa (primary PIN). This differential kinetic evolution suggests that they represent biologically different conditions, although with similar morphology. High-grade PIN is a neoplasm of uncertain biologic behavior, which occurs as a primary disease but exists most often in asso-

Fig. 2 In situ end-labeling in a primary PIN, b secondary PIN, and c low-grade PCa. Very low signal was detected in ductal cells affected by either primary PIN or low-grade PCa, whereas significantly higher values were obtained in secondary PIN. Note the positive signal provided by stromal and endothelial cells in c (internal control). a, b ×400; c ×200
The kinetic features of secondary PIN are consistent with an evolved tumor cell stage with regressive features (low proliferation and high apoptosis). Actually, in situ genetic analysis of coexistent PIN and PCA foci often shows similar chromosomal anomalies, although several PIN foci have been demonstrated to have more alterations than matched carcinoma foci [39]. Preliminary results from a comparative genomic hybridization analysis of PIN from this series revealed more extensive genetic abnormalities in secondary PIN and the coexistent PCA (20q12, 12q14, 9q22, and 16p12) than in primary PIN (20q and 16p) [28]. Those findings suggest that PIN foci can undergo more extensive chromosome evolution than association with invasive PCAs; its clinicopathological correlates remain to be defined. The histological and biological information on high-grade PIN is based mainly on case series of secondary PIN, and there are no papers addressing the kinetic and biologic differences between primary and secondary PIN. Our results display two distinctive kinetic profiles for them. Primary PIN cases were evenly distributed around MIB-1 and ISEL thresholds (50% above and 50% below), whereas a decreasing PR (86% of cases had an MIB-1 index <3%) and increasing ISEL index (80% of cases showed an ISEL index >0.4%) characterized secondary PIN (Figs. 2, 4). The kinetic pattern of low-grade PCAs was the opposite of that observed in secondary PIN (relatively high proliferation and comparatively low apoptosis) and closer to that of primary PIN than to that of secondary PIN. When all these features are considered together, the most likely kinetic evolution of secondary PIN (Fig. 4) seems to be from primary PIN (divergent progression, invasive and intraductal) rather than from low-grade PCAs (linear progression, primary PIN → low-grade PCAs → secondary PIN).

Fig. 3a, b Primary PIN. Relatively low proliferation rate characterized PIN, as detected by nuclear staining with MIB-1 antibody. a H&E, ×250; b MIB-1, ×400

Fig. 4 DNA histogram patterns in a primary and b secondary PIN. Diploid G0/G1 cells were frequently found in primary PIN and low-grade PCAs, while secondary PIN revealed hyperdiploid G0/G1 cells (peak labeled 1 in both panels).
PCAs and would also support a potential multifocality in the evolution of PCAs [2, 3, 6]. Likewise, they point to an independent clonal evolution of secondary PIN cells, different from that of the sequence primary PIN → low-grade PCAs [28]. A progressive accumulation of genetic abnormalities in PIN would increase the probability of genetically lethal events in those tumor cells, thus rising the apoptosis rate. The increase in the cell loss determines a reduction in the pool of mitotically active cells and, therefore, lowers PR. However, this up-regulated apoptosis pattern is not unique to high-grade PIN and has also been described in ductal carcinoma in situ (DCIS) of the breast. Harn et al. [21] reported significantly higher ISEL indices in breast DCIS than in either invasive ductal carcinomas or metastatic breast carcinomas, strongly correlated with abnormal p53 expression in the intraductal component. Multiple deletions involving several tumor suppressor genes are relatively frequent findings in intraepithelial neoplasms [17, 20, 21, 41]. We therefore propose that a distinction be made between primary and secondary PIN, in a similar way to the distinction in terminology that is widely accepted in bladder pathology [37, 49, 50]. This would mean interpreting secondary PIN as part of the evolution of prostate carcinoma rather than as a precursor, as suggested in some clinicopathological studies [30, 42].

Our results also emphasize the key role of apoptosis in early prostatic neoplasms. Initially, the association of high-grade PIN with low-grade PCAs might be surprising, but previous reports have demonstrated it [38, 40] and we must consider that PIN grading is based on cytological features while Gleason grading relies only on architectural parameters and most PCAs show a high nuclear grade. Therefore, that transition is not so surprising, although the volume of PIN is positively correlated with Gleason score [40]. The apoptosis analysis, contrasted by DNA-ploidy and ISEL of fragmented DNA, showed extremely low indices in low-grade PCAs (80% of low-grade PCAs displayed ISEL index <0.4% and revealed the highest nuclear area/DNA index ratio), smaller than in primary PIN. At the kinetic level, these two conditions were proven to be statistically different from secondary PIN, essentially as the result of apoptosis down-regulation. Under physiologic control, apoptosis closely correlates with proliferation to maintain a constant cell number [27]. A continuous increase in proliferation markers normally defines tumor progression, with a parallel increase in apoptosis [43]. Prior to any significant difference in proliferation markers, intraepithelial lesions seem to down-regulate apoptosis, resulting in a kinetic advantage and monoclonal expansions specifically of these lesions, as has been shown in breast DCIS [12], benign adrenal cortical proliferative lesions [8, 13], and adrenal medullary hyperplasia [10, 16] or C-cell hyperplasias in MEN-2A [9, 14, 51]. Decreased apoptotic cell loss would increase the cellular pool and allow the accumulation of genetically damaged cells, ending in a convergent cellular selection. That selection process would explain both tumor progression and heterogeneity, whether or not related with androgen levels or with androgen receptor abnormalities [26, 32, 34, 35].

Previous reports showed a progressive and variable increase of apoptosis in PIN and PCAs, but with SDs equal to or higher than the average [18], precluding any statistical assessment. This finding has been proposed as an indication of the continuum, or pathway, leading to PCAs. However, apoptotic indices are normally low and require screening of several HPF to achieve reliable results. This is especially true for heterogeneous conditions, such as PCAs. That inherent heterogeneity determines group overlapping and makes it difficult (and sometimes impossible) to reach statistical conclusions.

Proliferation parameters have been shown to be useful in the diagnosis of intraepithelial lesions (distinction of atypical hyperplasias from carcinomas in situ) and directly correlated with tumor grading [5, 7, 23, 36]. Our proliferation results suggest a kinetic difference between primary and secondary PIN: only secondary PIN was proven to be statistically different from both primary PIN and low-grade PCAs, with higher scores in the latter conditions than in secondary PIN (Table 1). We have found a similar kinetic pattern in CIS associated with muscle invasive transitional cell carcinoma of the urinary bladder: significantly decreased proliferative indices were revealed in the intraepithelial compartment than in the invasive one [17].

Our results are in the range of grade-1 PCAs reported by Helpap [23], and both his and ours are lower than those reported by Tamboli et al. [45]. These last authors did not provide MIB-1 indices by tumor grade and stage, but all their tumors had Gleason’s scores of 5+, and over one-third of them were stage III–IV. That is a different series distribution from ours, which showed a combined Gleason score of ≤4. The MIB-1 indices previously reported for PIN are in the range of our scores in primary PIN but reveal high variability (the SD was 1.5-fold the corresponding average) [45], probably an expression of tumor heterogeneity. Both intratumoral heterogeneity and the quantitation method will contribute to these discrepant results. We screened 50 HPF/focus (or the complete lesion if smaller) and 5 different foci per patient and pathologic condition to decrease the score variability (the smaller the SD the higher the measurement accuracy) and to take the intratumoral heterogeneity into consideration. Our SD values were always below average, suggesting the use of a better sampling method for tumor evaluation than for previously reported results [23, 45]. Studies in other organs have demonstrated a topographic heterogeneity of kinetic features missed with the standard screening methods [11, 16].

In summary, two kinetic profiles could be drawn in PIN, depending on whether or not it was associated with coexistent low-grade PCAs, which suggests that these represent biologically different conditions. The unique kinetic profile of secondary PIN, with relatively low proliferation and relatively high apoptosis, is consistent with an evolved tumor cell stage with regressive features rather than with a precursor lesion. Early steps in tumorigen-
esis in low-grade prostate carcinomas are kinetically characterized by down-regulated apoptosis rather than by increased proliferation.

References

DNA in plasma samples by applying the same PCR protocol as Drago et al.2,3 sequenced the amplification product, and could detect HHV-7 sequences in only about 50% of the samples, as opposed to 100% in the reports by Drago et al. The search for antibodies showed the absence of IgM antibodies, as well as no increase in IgG titer (except for 1 case), both of which are expected to increase in primary and/or reactivation infections.

(2) Yoshida4 applied the same nested PCR protocol as Drago et al.2 to peripheral blood DNA of patients with PR and healthy individuals, and found a signal of equal intensity in all samples. This finding argues against an increase in viral load in the PBMCs of patients with PR and against the specific occurrence of the viral sequences in the PR samples. This finding seems to us that the association between HHV-7 and PR is not firmly established.

In their letter above, Drago et al raise the interesting issue of whether an infectious agent, or markers thereof, must, or must not, be constantly present in an acute lesion induced by a herpesvirus. Both our knowledge on the biology of herpesvirus infections and the investigative tools available today have changed so much that there is currently a need to establish clear-cut criteria for pathogenetic association and causality. To review these criteria is beyond the purpose of the present reply. At the risk of oversimplifying the problem, in general, during acute phases of infections with herpesviruses (primary or reactivation), either 1 or both of the following conditions seem to apply: (1) the virus is present in the lesion and/or (2) there is an increase in the antibody response. At the current state of the art, in our opinion, the key issue concerning PR is whether the available evidence does, or does not, support an association with HHV-7 infection or reactivation. Establishing a causative role should be a subsequent step. To establish an association, consistency of the association is a minimum requirement. Independent laboratories have failed to confirm the presence of HHV-7 in PR lesions, or to reveal serologic or immunologic markers of an active infection or reactivation in all patients with PR. At the current state of the art, even without taking into consideration our own study, it seems to us that the association between HHV-7 and PR is not yet firmly established.

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Regarding MM biology, tumor thickness, apart from determining prognosis, identifies 2 biologically different types of MM. This is not specific for MM, but applies generally to tumor pathology. Evidence from other organ systems including the uterine cervix (squamous cell carcinoma), endometrium or stomach (adenocarcinoma), and urinary bladder (transition cell carcinoma) reveals the same 2 main types of malignancies. In general, most tumor risk factors and true precancerous lesions that are susceptible to screening programs are mainly associated with malignancies of protracted natural history, which explains the unchanged incidence of high-grade neoplasms in those organs. These high-grade tumors normally develop de novo and show no associated precancerous lesions, as expressed in thick MMs that are frequently nodular and mostly fast-growing tumors. This sort of tumor is not susceptible to any screening programs that are mainly associated with malignancies of protracted natural history, which explains the unchanged incidence of high-grade neoplasms in those organs. These high-grade tumors normally develop de novo and show no associated precancerous lesions, as expressed in thick MMs that are frequently nodular and mostly fast-growing tumors. This sort of tumor is not susceptible to any screening, which explains the failure of efficient early detection programs.1 This equally answers the open question that is the editorial’s title: no current screening program will effectively address the early diagnosis of thick nodular MM in a potentially curable stage. Unfortunately, our technology does not yet offer this possibility. Only the advent of new diagnostic techniques to detect subtle genetic or functional changes of early transformed melanocytes can offer a better prognosis for patients with nodular MM.

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An Alternate Explanation for the Increase in the Incidence of Melanoma Being Restricted to Patients With Thin Lesions

Drs Lipsker et al1 recently reported that in a carefully conducted population-based study there was a dichotomy between the rising incidence of thin melanomas and the stable incidence of thicker lesions. The authors point out that the excision of increasing numbers of thin melanomas had no effect on the incidence of thick melanomas. They propose as an explanation that thin and thick melanomas have different epidemiological features, and that the increased incidence of this cancer is owing to the recognition of a thin form of melanoma that is innocuous and unlikely to cause death if not treated.

However, there is an alternate interpretation that leads to a different conclusion. It flows from a simple mathematical analysis of the differing impacts of (1) the increasing incidence of melanoma, and (2) early detection on the proportion of patients who will have thin as opposed to thick lesions at diagnosis. It is illustrated by the following example: Assume that at baseline there are 100 new melanomas per year in a given population, and that 50% of these are diagnosed while still thin; 50 thin melanomas will then be diagnosed per year. Suppose that at a later date the incidence of melanoma has doubled, but that as a result of earlier detection the proportion diagnosed while still thin improves by half to 75%. The incidence of melanoma will now be 200 new cases per year, 75% (150) of which will be thin, and the number of thick melanomas will remain at 50 per year. Thus, a doubling in the incidence of melanoma coupled with a 50% improvement in early diagnosis results in a tripling of cases diagnosed while still thin, with no changes in the number of thick lesions.

The data presented by Drs Lipsker et al1 is remarkably consistent with this interpretation. The number of new melanomas in Strasbourg, France, more than doubled between 1980 and 1997, from fewer than 40 per year to more than 90 per year (see their Figure 2). During the same time the number of melanomas diagnosed while still thin increased 4-fold from less than 15 per year to approximately 60 per year (see their Figures 6 and 7), while that of thick melanomas increased only slightly.

Are these different interpretations of more than academic importance? Yes, because their implications are very different. That of Drs Lipsker and colleagues1 implies that the effort to reduce melanoma mortality by early detection and treatment is ineffective because the lesions being detected and removed would not have caused death in any event. By contrast, the interpretation pro-

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posed here implies that this effort is effective, as evidenced by the increasing proportion of melanomas diagnosed while still thin and at a stage when they are completely curable. The fact that the number of thick melanomas is not decreasing reflects that the incidence of this cancer is increasing more rapidly than our ability to detect it early. The implication is that efforts at early diagnosis should be intensified and coupled with an equally strong effort to reduce the incidence of this cancer.

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In reply

We thank Dr Bystryn for his interest in our recently published work in this journal. Dr Bystryn proposes an alternate explanation for the increase in the incidence of thin melanomas. His explanation relies on a mathematical analysis of the differing impacts of increasing incidence and early detection. According to his model, the combined effect of an overall increase in melanomas and simultaneous improvement in early detection of thin lesions could lead to a stable number of thick melanomas (because their proportion decreases) and an increase in the number of thin melanomas (the proportion of which increases). We agree that the increase in incidence of thin melanomas is in part related to better and earlier detection of melanomas. We also agree that early detection is the best treatment of melanoma and remains essential.

However, we do not think that this mathematical analysis explains the difference we observed between incidence rates of thick and thin melanomas. Indeed, this model implies 3 postulates, which Dr Bystryn did not address. First, it supposes that every thick melanoma is the result of a thin melanoma. Second, it supposes that the time lapse during which a thin lesion becomes a thick lesion is long enough to allow efficient detection at the thin stage in all cases. That would mean that this time is compatible with detection and that people would consult with a physician during this period. Third, it supposes that the whole population would be screened. If any of these postulates would not hold true, improvement of detection alone could not explain the stability in the incidence of thin melanomas while there is a steady increase in thick melanomas.

Our data support the notion that the lapse of time during which thin tumors become thick tumors is not always long enough to allow detection. Indeed, this time lapse can be very short and was less than 3 months in some patients with thick, fast-growing, nodular melanomas. Furthermore, our data showed a constant and regular increase in the incidence of thin melanomas over 18 years, while the incidence of thick melanomas remained stable during this period. According to Dr Bystryn’s model, this would mean that there was a proportional, year-by-year improvement of detection during this period. However, until 1998 there was no organized detection of melanoma in the Bas-Rhin region of France. Furthermore, we showed in a Strasbourg University Hospital–based study that the proportion of melanomas diagnosed by means of detection was extremely low until the 1990s, while incidence of thin melanomas had already doubled by this time compared with 1980. Even though detection is essential, its efficiency, eg, the percentage of the population that is actually screened, probably remains low in the Bas-Rhin and can still be improved. Thus, it probably does not explain why incidence rates of thick tumors remained stable during this period. Therefore, and although the mathematical analysis suggested by Dr Bystryn may account for part of the increase in thin melanomas, further study should be conducted to clarify why the evolution of incidence rates of thick and thin melanomas do not seem to be related.

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VIGNETTES

Treatment of Labial Lentigos in Atopic Dermatitis With the Frequency-Doubled Q-Switched Nd:YAG Laser

Recently, atopic dermatitis has been increasing throughout the world, and there have been many labial lentigos caused by a postinflammation of atopic dermatitis. Laser therapy, such as the normal ruby laser and the Q-switched ruby laser, is one of the treatment methods. We make the first report of 4 cases of labial lentigos in atopic dermatitis treated with the frequency-doubled Q-switched Nd:YAG laser at a wavelength of 532 nm. We achieved rapid results and a dramatic clearing of the lesions without cutaneous alterations in skin texture.

Patients and Methods. We used the frequency-doubled Q-switched Nd:YAG laser (Continuum Biomedical Inc, Livermore, Calif), which has a pulse duration of 5 to 7 nanoseconds, a wavelength of 532 nm, a pulse repetition rate of 10 Hz, and a spot size of 3 mm. Local anesthesia using 60% lidocaine tape was placed on the affected part 1 hour before laser irradiation. After laser irradiation, 0.12% betamethasone valerate ointment containing 0.1% gentamicin sulfate was applied for 3 days. All subjects had labial lentigos with atopic dermatitis. No
The correlation of regression with the grade of dysplasia (atypia) in melanocytic naevi

Sir: The diagnosis of atypical melanocytic naevus (AMN) is controversial, which partly explains its variable incidence. The AMN consensus definition includes the presence of lymphocytic inflammation, which is known to induce signs such as colour changes, swelling and erythema, considered to be criteria of atypical moles. The inflammation seen in naevus regression seems to be reactive to unidentified melanocytic changes, but its relationship to AMN grading remains unknown.

We studied 123 clinically atypical naevi from 65 female and 46 male subjects aged 36.56 ± 11.94 years. No significant differences were observed associated with regression regarding age and sex distribution, location, gross appearance, family or personal history of atypical mole syndrome/malignant melanoma. Cases showing evidence of scarring, incomplete resection or features suggesting congenital onset were excluded.

Multiple haematoxylin and eosin-stained sections of the whole lesion were evaluated by two independent observers (A.B. and S.J.D.-C.). In cases of disagreement, grading was assigned during simultaneous inspection. Reproducibility data were not recorded. AMN were required to show both architectural and cytological atypia, including lentiginous melanocytic hyperplasia (discontinuous/confluent), suprabasilar melanocytes (little/fully pagetoid spread), nesting variation and

**Figure 1.** Low- and high-grade atypical (dysplastic) melanocytic naevi (AMN). A, scanning view of a low-grade AMN with regression. It shows a dense lymphohistiocytic inflammatory infiltrate with numerous melanophages (arrow), irregularly distributed melanocyte clusters, focal fibrosis and prominent blood vessels. (H&E.) A high-power view reveals melanocytes with round-to-oval nuclei, smooth chromatin and small and single nucleoli (H&E).

B, low-power view of a high-grade AMN showing a mixed (nested and lentiginous) junctional proliferation of melanocytes. Note the presence of prominent blood vessels and the absence of melanophages and significant fibrosis. (H&E.) High-power view reveals melanocytes with irregular nuclear contours (marked pleomorphism), hyperchromatism, or with prominent nucleoli. Anisokaryosis is also observed. (H&E.)

bridging (little/confluent nests), anisokaryosis (size variation $\geq 3:1$), nuclear pleomorphism, prominent nucleoli and hyperchromatism.\textsuperscript{2,4} Variables were independently evaluated and considered positive if present in $\geq 50\%$ of the lesion. AMN were scored according to the number of criteria fulfilled into mild (two to three criteria), moderate (four to five criteria) and severe (six to seven criteria). Side-to-side junctional symmetry was evaluated according to the patterns (lentiginous/nested) on both sides, the same for symmetric and different for asymmetric naevi. We also analysed kinetic features (proliferation by Ki67 index and apoptosis by in situ end labelling) of these lesions in two topographical compartments (junctional and dermal). Statistical differences regarding the presence of regression were analysed by Fisher’s exact tests and analysis of variance (significant if $P < 0.05$).

Regression, defined by both junctional and dermal melanocyte dropout, dermal non-lamellar fibrosis, increased dermal vascularity and a variable degree of lymphocytic infiltrate with melanophages (Figure 1), was confirmed by the lowest proliferation/apoptotic index in the dermal compartment and was significantly associated with mild AMN and moderate AMN (Figure 2). Only prominent nucleoli and anisokaryosis showed no statistically significant differences in AMN whether or not associated with regression. Given the high incidence and frequent coexistence of these features, reliable diagnosis of severe AMN with regression requires marked pleomorphism, hyperchromatism and at least one other nuclear and architectural abnormality. Additionally, regression of inflammation would be responsible for an ill-defined border, irregular pigmentation and erythema, thus contributing to the atypical clinical picture of mild and moderate AMN.\textsuperscript{3}

The inverse correlation between regression of inflammation and degree of dysplasia suggests that atypia could be incidental to regression of inflammation and proliferation.\textsuperscript{5,6} Inflammation-induced apoptosis results in nuclear atypia and, under these circumstances, the active radial growth phase could be reactive to that regression. Scenarios such as an inflammatory response reactive to melanocyte neoplastic transformation or a common initiator for both melanocyte dysplasia and
CD10 is a characteristic marker of tumours forming morules with biotin-rich, optically clear nuclei that occur in different organs

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Sir: Recently, Chiarelli et al. have demonstrated that CD10 immunoreactivity represents a useful marker of morules in endometrioid lesions of the female genital tract, allowing identification of various low-grade lesions which are associated with these nodular structures. Cytologically, morules exhibit bland cells lacking intercellular bridges and keratinization and express neither high-molecular-weight keratins nor involucrin. They are not related to human papillomavirus infection and are thus different from both squamous metaplasia and carcinoma. Morules with a characteristic nuclear clearing have been reported in endometria, ovarian endometrioid tumours, colorectal adenomas and carcinomas, gastric polyps, gallbladder adenomas, pulmonary blastomas, low-grade adenocarcinomas of fetal lung type, some papillary lung carcinomas, pancreaticoblastomas and, finally, in the cribriform-morular variant (C-MV) of papillary thyroid carcinomas. The so-called ‘peculiar nuclear clearing’ so frequently found in these morules is due to biotin-rich intranuclear inclusions which ultrastructurally have the appearance of thread-like fibrils and consequently should not be misinterpreted as intranuclear cytoplasmic pseudo-inclusions or as viral inclusion bodies.

In order to demonstrate the usefulness of CD10 in the diagnosis of morphologic metaplasia in neoplasms in extragenital locations, the immunoreexpression of CD10 and β-catenin was studied in seven cases of tumours containing morules with cells displaying biotin-rich, optically clear nuclei (BROCN; BROCN-family tumours), corresponding to four instances of CM-V of papillary thyroid carcinoma (one of them in the setting of familial adenomatous polyposis), one case of low-grade adenocarcinoma of fetal lung type, one case of pulmonary blastoma and one pancreaticoblastoma in a child with Beckwith–Wiedemann syndrome (Figure 1). Immunohistochemistry was performed on paraffin sections using a universal secondary antibody kit that used a peroxidase-conjugated labelled-dextran polymer (Dako EnVision Peroxidase/diaminobenzidine: Dako, Glostrup, Denmark), in order to avoid misinterpreting endogenous biotin or biotin-like activity in the cell cytoplasm or nuclei as immunopositivity. All cases revealed strong CD10 membranous positivity (clone 56C6, dilution 1:10, microwave oven, Tris—ethylenediamine tetraacetic acid: NovoCastra, Newcastle-upon-Tyne, UK) in morules (Figure 1), with some cytoplasmatic staining, probably due to diffusion. As a result, CD10 positivity allowed easy identification of morules at low power in the different samples. CD10 immunopositivity also outlined the luminal borders of the neoplastic cells in the glandular formations of all tumours. Aberrant nuclear expression of β-catenin (β-catenin-1, 1:300, microwave oven; Dako) was