Clonal Composition of Human Adrenocortical Neoplasms

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ABSTRACT

The mechanisms of tumorigenesis of adrenocortical neoplasias are still not understood. Tumor formation may be the result of spontaneous transformation of adrenocortical cells by somatic mutations. Another factor stimulating adrenocortical cell growth and potentially associated with formation of adrenal adenomas and, less frequently, carcinomas is the chronic elevation of proopiomelanocortin-derived peptides in diseases like ACTH-dependent Cushing's syndrome and congenital adrenal hyperplasia. To further investigate the pathogenesis of adrenocortical neoplasias, we studied the clonal composition of such tumors using X-chromosome inactivation analysis of the highly polymorphic region Xcen-Xp11.4 with the hybridization probe M2B7, which maps to a variable number of tandem repeats on the X-chromosome. In addition, polymerase chain reaction amplification of a phosphoglycerokinase gene polymorphism was performed. After DNA extraction from tumorous adrenal tissue and normal leukocytes in parallel, the active X-chromosome of each sample was digested with the methylation-sensitive restriction enzyme HpaII. A second digestion with an appropriate restriction enzyme revealed the polymorphism of the region Xcen-Xp11.4 and the phosphoglycerokinase locus. Whereas in normal polyclonal tissue both the paternal and maternal alleles are detected, a monoclonal tumor shows only one of the parental alleles. A total of 21 female patients with adrenal lesions were analyzed; 17 turned out to be heterozygous for at least one of the loci. Our results were as follows: diffuse (n = 4) and nodular (n = 1) adrenal hyperplasia in patients with ACTH-dependent Cushing's syndrome, polyclonal pattern; adrenocortical adenomas (n = 8), monoclonal (n = 7), as well as polyclonal (n = 1); and adenocarcinomas (n = 3), monoclonal pattern. One metastasis of an adrenocortical carcinoma showed a pattern most likely due to tumor-associated loss of methylation. In the special case of a patient with bilateral ACTH-independent macroadrenocortical hyperplasia, diffuse hyperplastic areas and a small nodule showed a polyclonal pattern, whereas a large nodule was monoclonal. We conclude that most adrenal adenomas and carcinomas are monoclonal, whereas diffuse and nodular adrenal hyperplasias are polyclonal. The clonal composition of ACTH-independent massive macronodular hyperplasia seems to be heterogeneous, consisting of polyclonal and monoclonal areas.

INTRODUCTION

The pathogenesis of cancer is generally a multistep process, during which an initiation event is followed by tumor promotion. The initiation event is widely regarded as a somatic mutation occurring in a single cell, which, because of a selective growth advantage, proliferates to produce a monoclonal tumor (1, 2). Adrenocortical steroid secretion is a complex process that is regulated by several hormones and growth factors. The hormonal control of glucocorticoid, mineralocorticoid, and sex steroid secretion also regulates adaptive processes like hypertrophy and hyperplasia of the adrenal cortex (3, 4). Diffuse and nodular adrenal hyperplasia has been observed in diseases associated with chronic elevation of proopiomelanocortin-derived peptides like ACTH-dependent Cushing's syndrome (5) and congenital adrenal hyperplasia (6). Besides spontaneous transformation of adrenocortical cells by somatic mutations, adrenocortical tumor formation may, therefore, be the result of chronic stimulation of the adrenal cortex.

According to Lyon's (7) hypothesis, 1 of the 2 X-chromosomes in all female somatic cells is functionally inactivated early in embryogenesis by changes in the methylation pattern of cytosine residues. This random process is fixed for a given cell and its progeny. Therefore, a roughly equal mixture of 2 types of precursor cells gives rise to polyclonal female tissue, because some contain an active maternal, some an active paternal X-chromosome (8). In contrast, in a neoplasm derived from a single somatic cell, all the tumor cells would retain the same X-inactivation pattern. In 1985, Vogelstein et al. (9) reported the possibility of determining clonality of human tumors based on RFLP3 of X-chromosome-linked genes like PGK or hypoxanthine phosphoribosyltransferase. Since these RFLPs are bimorphisms, constitutional heterozygosity may at best be 50%, but it is frequently lower. Therefore, many patients are constitutionally homozygous and uninformative for assessment of tumor clonality by X-chromosome inactivation analysis. The discovery of the highly polymorphic locus DXS255 by Fraser et al. (10) was a great advance because it allowed overcoming of the limitations of X-linked RFLPs. This X-linked locus (Xcen-Xp11.4) shows a rate of heterozygosity of more than 90% due to the presence of a variable number of tandem repeats (10–14). Recently, Gilliland et al. (15) developed a new polymerase chain reaction-aided method using the polymorphism of PGK, but having the advantage of much greater sensitivity (as few as 100 cells should be enough to make a distinction between monoclonal and polyclonal tissue) and avoiding the laborious Southern blot techniques.

In this study, we investigated the clonal composition of diverse types of adrenocortical neoplasms from 21 unrelated female patients with diverse adrenocortical neoplasms.

PATIENTS AND METHODS

Patients. Twenty-one informed and consenting female patients undergoing adrenalectomy for various adrenocortical diseases were analyzed for heterozygosity of the region Xcen-Xp11.4 and the PGK locus. Patients were diagnosed by standard clinical, biochemical, imaging, and histological criteria. The clinical data are summarized in Table 1. The protocol was approved by the ethics review committees of the University of Würzburg and the National Institute of Child Health and Human Development.

Tissue and DNA Preparation. Sources of normal DNA were whole blood leukocytes and, whenever possible, normal adrenal or other normal tissues. Tumors and tissue samples were frozen in liquid nitrogen immediately after surgery and stored at −80°C. To avoid contamination with surrounding tissue (like capsule- or fat tissue), nonneoplastic areas were removed macroscopically and only a central part of each tissue specimen was used. DNA was isolated from blood and tissues, as described previously (16). After quantification of the DNA content by photometric measurement, an undigested aliquot of the DNA was digested with a restriction enzyme to linearize the DNA. A total of 21 female patients with adrenal lesions were analyzed; 17 turned out to be heterozygous for at least one of the loci. Our results were as follows: diffuse (n = 4) and nodular (n = 1) adrenal hyperplasia in patients with ACTH-dependent Cushing's syndrome, polyclonal pattern; adrenocortical adenomas (n = 8), monoclonal (n = 7), as well as polyclonal (n = 1); and adenocarcinomas (n = 3), monoclonal pattern. One metastasis of an adrenocortical carcinoma showed a pattern most likely due to tumor-associated loss of methylation. In the special case of a patient with bilateral ACTH-independent macroadrenocortical hyperplasia, diffuse hyperplastic areas and a small nodule showed a polyclonal pattern, whereas a large nodule was monoclonal. We conclude that most adrenal adenomas and carcinomas are monoclonal, whereas diffuse and nodular adrenal hyperplasias are polyclonal. The clonal composition of ACTH-independent massive macronodular hyperplasia seems to be heterogeneous, consisting of polyclonal and monoclonal areas.

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3 The abbreviations used are: RFLP, restriction fragment length polymorphism; PGK, phosphoglycerokinase; PCR, polymerase chain reaction.
aliquots were double digested further with either MspI or HpaII, respectively, at an nonsensitive isoschizomer of HpaII)-treated samples demonstrating the maternal and paternal X-chromosomes. The PstI/MspI (a methyl

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DNA was transferred by capillary elution to nylon membranes, cross-linked by ultraviolet light (Gene Linker; Bio-Rad, München, Germany), prehybridized for 20 min at 68°C with Quickhyb solution (Stratagene, Heidelberg, Germany), and hybridized for 1 h at 68°C in 10 ml hybridization solution containing salmon sperm and the described methods (15) with minor modifications. In brief, leukocytes and 32P-labeled enzymes were purchased from Amersham/USB (Braunschweig, Germany). The probe was labeled with [32P]dCTP using a random primer labeling kit and Amplification of the PGK Locus. Investigation of the clonal composition

Hybridization, Detection, and Interpretation. DNA was transferred by capillary elution to nylon membranes, cross-linked by ultraviolet light (Gene Linker; Bio-Rad, München, Germany), prehybridized for 20 min at 68°C with Quickhyb solution (Stratagene, Heidelberg, Germany), and hybridized for 1 h at 68°C in 10 ml hybridization solution containing salmon sperm and the 32P-labeled M27B probe in a hybridization oven. After hybridization, the membranes were washed twice in 1X standard saline-citrate, 0.5% sodium dodecyl sulfate at room temperature, and then again 30 min at 60°C. Kodak X-OMAT DS films were exposed for autoradiography using intensifying screens at −80°C for 12 h to 4 days. To detect the DXS255 locus, we used the M27B probe, which was kindly provided by Dr. Ian Craig and Dr. Neil Fraser. The probe was labeled with [32P]dCTP using a random primer labeling kit following the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany).

The PstI-digested samples show 2 bands in heterozygous patients, representing the maternal and paternal X-chromosomes. The PstI/MspI (a methylatation-sensitive isoschizomer of HpaII)-treated samples demonstrate the complete digestion of both the methylated and unmethylated allele resulting in 2 bands, whereas the PstI/HpaII (that distinguishes the active and inactive allele) digestion gives rise to 4 bands in polyclonal and 2 bands in monoclonal tissue.

Amplification of the PGK Locus. Investigation of the clonal composition using the PCR amplification of the PGK locus was performed using previously described methods (15) with minor modifications. In brief, leukocyte and tumor DNA samples were split into halves and incubated in the presence or absence of HpaII. One μl of HpaII-digested or nondigested samples was used for amplification using only the internal primers of the original protocol. The

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yrs)</th>
<th>Clinical presentation</th>
<th>Hormonal status</th>
<th>Histology of adrenocortical neoplasia</th>
<th>Ratio*</th>
<th>Clonality</th>
<th>PGK locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>Ectopic ACTH syndrome</td>
<td>Hypercortisolism</td>
<td>Diffuse and focal nodular hyperplasia</td>
<td>1.48:1.33</td>
<td>P⁶</td>
<td>P</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>Pituitary-dependent Cushing’s syndrome</td>
<td>Hypercortisolism</td>
<td>Bilateral diffuse hyperplasia</td>
<td>2.05:1.54</td>
<td>P</td>
<td>—</td>
</tr>
<tr>
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<td>52</td>
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<td>Hypercortisolism</td>
<td>Diffuse hyperplasia</td>
<td>1.07:1.95</td>
<td>P</td>
<td>P</td>
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<td>Diffuse hyperplasia</td>
<td>1.92:1.48</td>
<td>P</td>
<td>—</td>
</tr>
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<td>5</td>
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<td>Pituitary-dependent Cushing’s syndrome</td>
<td>Hypercortisolism</td>
<td>Bilateral nodular hyperplasia; dominant nodule (Ø 2 cm)</td>
<td>3.30:2.03</td>
<td>P</td>
<td>—</td>
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<td>6</td>
<td>53</td>
<td>Conn’s syndrome</td>
<td>Primary hyperaldosteronism</td>
<td>Adrenal adenoma (Ø 3 cm)</td>
<td>3.30:1.48</td>
<td>P</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>49</td>
<td>Adrenal Cushing’s syndrome</td>
<td>Hypercortisolism</td>
<td>Adrenal adenoma (Ø 3 cm)</td>
<td>1.18:1000</td>
<td>M</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
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<td>Primary hyperaldosteronism</td>
<td>Adrenal adenoma (Ø 3 cm)</td>
<td>2.19:6.34</td>
<td>P</td>
<td>P</td>
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<td>51</td>
<td>MEN 1, adrenal Cushing’s syndrome, virilization</td>
<td>Hypercortisolism, hyperandrogenism</td>
<td>Metastatic adrenal carcinoma; liver metastasis (Ø 9 cm)</td>
<td>3.30:2.03</td>
<td>P</td>
<td>—</td>
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<tr>
<td>10</td>
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<td>Asymptomatic incidentaloma</td>
<td>Nonfunctional</td>
<td>Adrenal adenoma (Ø 2 cm)</td>
<td>1.13</td>
<td>P</td>
<td>—</td>
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<tr>
<td>11</td>
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<td>Asymptomatic incidentaloma</td>
<td>Nonfunctional</td>
<td>Adrenal adenoma (Ø 5 cm)</td>
<td>1:1.5</td>
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<td>M</td>
</tr>
<tr>
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<td>Primary hyperaldosteronism</td>
<td>Adrenal adenoma (Ø 9 cm)</td>
<td>1:2.2</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>13</td>
<td>24</td>
<td>Cushing’s syndrome</td>
<td>Hypercortisolism</td>
<td>Metastatic adrenal carcinoma; lung metastasis (Ø 0.5 cm)</td>
<td>3.30:2.03</td>
<td>P</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>32</td>
<td>ACTH-independent Cushing’s syndrome</td>
<td>Hypercortisolism</td>
<td>Bilateral macronodular hyperplasia (Ø 1–3.5 cm)</td>
<td>1.13</td>
<td>P</td>
<td>—</td>
</tr>
</tbody>
</table>

* Allelic cleavage ratio of WBCs or normal tissue to neoplastic tissue; Ø, diameter.

P⁶, polyclonal; M, monoclonal; —, noninformative; NA, not available/not analyzed; LOM, loss of methylation.

Normal allelic cleavage ratio values of whole blood leukocytes were reported to be <3.3. However, in some instances skewed values >3.3 have been described (37).
CLONAL COMPOSITION OF ADRENAL TUMORS

Fig. 1. Polyclonal pattern of a diffuse adrenocortical hyperplasia (Lanes d-f) and monoclonal pattern of an adenoma (Lanes j-l) at the DXS255 locus using the M27@ probe; leukocyte DNA of the corresponding patients are shown in Lanes a-c and g-i, respectively. kb, kilobase.

PCR product was after phenol chloroform/chloroform extraction and ethanol/sodium acetate precipitation digested with Bst XI, which reveals the polymorphism. Whereas in monoclonal tissue only one allele is present, polyclonal tissue gives rise to 2 bands.

Rate of Heterozygosity. Leukocyte DNA from 21 patients undergoing adrenalectomy for adrenocortical neoplasm was screened for the presence of heterogeneous polymorphisms in the DXS255 and/or PGK locus. Seventeen of those patients turned out to be heterozygous for either the DXS255 (n = 14 of 18; 78%) or the PGK (n = 7 of 21; 33%) locus. Four patients showing heterogeneous pattern for both loci were examined by both methods, resulting in identical clonal compositions. Three patients could not be further studied at the DXS255 locus, as insufficient amounts of intact high molecular weight tumor DNA were available.

Determination of Allelic Cleavage Ratio. Autoradiographs were scanned with a Densitometer Model Bio-Profil, Vilber Lourmat (Mone la Vallée, France). To determine the allelic cleavage ratio each HpaII band was compared to its homologous PstI band according to the following formula (17):

\[
\frac{\text{Upper PstI band} \times \text{lower HpaII band}}{\text{Upper HpaII band} \times \text{lower PstI band}}
\]

Reciprocal values were reported when the ratio was <1.0.

RESULTS

Adrenocortical Hyperplasia in ACTH-dependent Cushing's Syndrome. Four patients with diffuse hyperplasia and one patient with macronodular hyperplasia were analyzed. The diffuse adrenal hyperplasia samples showed a polyclonal pattern by Southern blot and PCR-aided techniques (Fig. 1 and Table 2). In the patient with macronodular hyperplasia, the diffuse hyperplastic area and one dominant nodule were studied separately. Both samples showed a polyclonal pattern (Fig. 3).

Adrenocortical Adenoma. Eight adrenocortical adenomas (3 aldosterone-producing adenomas, 3 cortisol-producing adenomas, and 2 nonfunctional adenomas) were analyzed. Five of them were informative for the DXS255 locus, 5 for the PGK gene, and 2 of the latter for both loci. Seven adenomas showed a monoclonal pattern (Figs. 1 and 2), whereas one cortisol-producing adenoma (Patient 7) was interpreted as a polyclonal tumor by both techniques (Fig. 4). The microscopic appearance of this tumor was that of a typical cortisol-producing adenoma and consisted of zona fasciculata-type and zona reticularis-type cells. The ipsilateral-adjacent cortex was atrophic. The tumor did not show any unusual histological features different from other cortical adenomas of this series, which would explain why it was polyclonal.

Adrenocortical Carcinoma. Three adrenocortical carcinomas (one lung metastasis, one very large liver metastasis, and one carcinoma itself) were examined for clonality by using the polymorphism of the DXS255 locus. The carcinoma and liver metastasis turned out to be monoclonal, whereas the lung metastasis (Patient 15) showed a "monoclonal" pattern most likely due loss of methylation at the DXS255 locus (Fig. 5).

ACTH-independent Macronodular Hyperplasia. The case of an ACTH-independent bilateral macronodular hyperplasia (Patient 17) heterozygous for the RFLPs at the DXS255 locus was extensively
studied. The right adrenal gland showed diffuse hyperplastic areas and many small nodules (up to 1.2 cm in diameter), whereas in the left hyperplastic adrenal cortex one dominant nodule of 3.5 cm in diameter and one smaller 0.4-cm nodule were present (Fig. 6, top). Histologically each of the nodules showed a mixture of lipid-rich and eosinophilic compact cells. The diffuse hyperplastic areas and one small nodule from the right adrenal were polyclonal, whereas a central part of the dominant nodule of the left adrenal gland was monoclonal (Fig. 6, bottom).

DISCUSSION

Adrenocortical adenomas are very frequent and can be found in 1.41–8.7% of unselected autopsies (18). Most of these tumors are clinically silent and are incidentally detected by ultrasound or computed tomography performed for unrelated reasons (19). The pathogenesis of these neoplasms is still not understood. Some investigators believe that extraadrenal factors like chronic exposure of the adrenal cortex to proopiomelanocortin-derived peptides in congenital adrenal hyperplasia may account for the development of adrenal neoplasms (4). On the other hand, there is overwhelming evidence that cancer is generally the result of somatic mutations, arguing for clonal expansion of a single transformed cell (20).

In this study, we examined the pathogenesis of adrenocortical neoplasms by determining their clonal composition. This approach implies that monoclonal adrenocortical neoplasms arise from a single transformed cell, whereas polyclonal adrenal tumors would suggest adrenal hyperplasia through extraadrenal stimuli. As our results show, the ACTH-dependent diffuse adrenocortical hyperplasia represents polyclonal adrenal tissue in accordance with chronic stimulation of the adrenal cortex. Due to its polyclonal pattern, the macronodular form of ACTH-dependent adrenal hyperplasia is probably a later stage of diffuse hyperplasia, developing under the influence of longstanding endogenous hyperadrenocorticotropinism (4). Similar results have been obtained in patients with multinodular goiters, whose diffuse hyperplastic areas of the thyroid and small thyroid nodules showed a polyclonal pattern. However, larger thyroid nodules in goiters were monoclonal, arguing for autonomous and adenoma-like growth characteristics (21). Similar findings with development of autonomous adenomas have been described in some patients with ACTH-dependent Cushing’s syndrome, in whom biochemical evaluation showed a transition from pituitary-dependent to non-ACTH-dependent Cushing’s syndrome (22, 23).

Our finding of monoclonality in nonfunctional and cortisol- and aldosterone-producing adrenocortical adenomas is strong evidence for a unicellular adrenal origin of those neoplasms. So far, mutations in several oncogenes have been described in adrenal adenomas. Constitutively activating point mutations of the α-chain of Gs, the inhibiting G-protein, were reported in 3 of 11 adrenocortical neoplasms (24).

However, in a larger series of patients, no such Gs2 mutations were found (25). Recently, Lin et al. (26) described mutations in the p53 tumor supressor gene in adrenal adenomas. Most of the mutations were heterozygous and affected codons not commonly found in other tumors, especially benign ones. p53 mutations are generally a late event in malignant tumorigenesis, and this questions the significance of p53 mutations reported in adrenal adenomas. In a previous study of p53 mutations in adrenocortical tumors, we found p53 mutations in 3 of 11 carcinomas and 2 of 2 adrenocortical cancer cell lines, but in none of 5 adenomas (27).

For the obviously polyclonal adrenaloma, several potential caveats must be discussed. Thus, improperly dissected tumor tissue may contain substantial amounts of surrounding tissue or stromal cells. As these nontumoral tissues are known to be polyclonal, this contamination could lead to the incorrect pattern of polyclonality. In addition, some neoplasms could have unusual methylation patterns through reactivation of some inactive X-chromosome alleles. This could also falsely suggest a polyclonal origin. Recently, Silva et al. (28) reported that methylation of a specific site in mammalian cell DNA is not strictly conserved and identical in progeny cells as the Lyon (7) hypothesis suggests, but reflects an equilibrium frequency defined by a continual loss and gain of methyl groups. It has also been reported that certain human tumor cell lines respond to cytotoxic chemotherapy or radiotherapy by DNA hypermethylation (29), and this could lead to misleading results in clonal investigation (30). In our case (Patient 7), the tumor was large enough (4 cm in diameter) to avoid contamination with adjacent capsule tissue. Additionally, this tumor was examined for clonality at 2 different X-chromosomal loci, which would be unlikely to be both affected by random demethylation through neoplastic growth.

Interestingly, the patient with a cortisol-producing adrenocortical

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**Fig. 4.** Polyclonal composition of a cortisol-producing adrenocortical adenoma. Left: DXS255; leukocyte DNA (Lanes a–c), adenoma (Lanes d–f); right: PGK locus; leukocyte DNA (Lanes g and h), adenoma (Lanes i and j). kb, kilobase.

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**Fig. 5.** Monoclonal pattern of an adrenocortical carcinoma (Lanes a–c) and the pattern of a methylation loss in the DXS255 locus in a lung metastasis (Lanes d–f). kb, kilobase.

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4930
adenoma in the context of multiple endocrine neoplasia type I showed a monoclonal pattern, which was similar to the monoclonal composition of parathyroid adenomas in this entity (31). The monoclonal pattern of adrenocortical carcinomas is not surprising, since these neoplasms are believed to be the result of somatic mutation(s). Mutational changes found in adrenocortical carcinomas affect the chromosomal loci 11q13, 11p15.5, and 17p13 (32). 11q13 is the locus of multiple endocrine neoplasia type I gene, and adrenal tumors are part of this hereditary tumor syndrome (33, 34). The Beckwith-Wiedemann syndrome, a rare familial tumor syndrome, is characterized by paternal disomy of 11p15.5 and rearrangements of the IGF II gene (35). Affected family members are at risk for development of nephroblastoma and adrenocortical carcinoma. The p53 tumor suppressor gene maps to 17p13 and p53 mutations have been described in adrenocortical cancer cell lines and carcinomas (27).

The case of a lung metastasis caused by metastatic adrenocortical carcinoma with loss of methylation in DXS255 locus shows the complexity of tumor growth and treatment. Prior to obtaining the tumor sample for study, the patient had undergone various chemotherapeutic protocols, including polychemotherapy, mitotane, and gossypol (36), which may have affected the methylation pattern in this tumor.

ACTH-independent bilateral macronodular hyperplasia is a rare cause of adrenal Cushing’s syndrome (37). It is characterized by low plasma ACTH concentrations in the presence of hypercortisolism nonsuppressible by dexamethasone. The radiological features include bilateral hyperplasia with multiple nodules of different size. Microscopic examination shows besides the nodules diffuse hyperplasia of the “normal” adrenal cortex. The etiopathology has so far not been elucidated. A subgroup of these patients apparently has food-dependent Cushing’s syndrome due to expression of the gastric inhibitory peptide receptor in the adrenal gland (38). In our patient, the adrenal cortex showed areas of diffuse hyperplasia, micronoduli up to 1 cm in diameter, and a dominant nodule in one adrenal gland. The diffuse hyperplastic area and the small nodule showed a polyclonal pattern, whereas the large was monoclonal. This may indicate transition from hyperplasia to autonomous adenoma-like growth characteristics in larger nodules of this entity.

ACKNOWLEDGMENTS

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ADDENDUM


REFERENCES
