Genetic Aberrations in Adrenocortical Tumors Detected Using Comparative Genomic Hybridization Correlate with Tumor Size and Malignancy

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ABSTRACT

The differentiation between malignant and benign adrenocortical tumors is often difficult, and better markers are required. Because the genetic background of adrenocortical tumors is poorly characterized, we used comparative genomic hybridization (CGH) to screen for DNA sequence copy number changes in 8 sporadic primary adrenocortical cancers and 14 adenomas. There was a strong relationship between the number of genetic aberrations detected using CGH and both tumor size and malignancy. No alterations were seen in the smaller adenomas (<5 cm), whereas the two largest adenomas (5 cm each) and seven of the eight cancers (7-20 cm) showed an increased number of genetic alterations. The presence of genetic aberrations detected using CGH was associated with an aneuploid DNA pattern. In the cancers, losses most often involved the chromosomal regions 2, 11q, and 17p (four of eight tumors), whereas gains took place at chromosomes 4 and 5 (four of eight tumors). In conclusion, our data indicate that genetic changes may help to define the malignant potential of adrenocortical tumors. Furthermore, the CGH results indicate several chromosomal regions that may contain genes with an important role in the development of adrenocortical cancers.

INTRODUCTION

Adrenocortical cancer is a rare malignancy, with an annual incidence of about 2 per million inhabitants a year according to the Swedish cancer registry. However, autopsy studies show that benign adrenocortical tumors are found in 2-9% of the population (1, 2). The interest in adrenocortical tumors has increased during the last decade, since tumors in the adrenals are found incidentally in more than 0.6% of patients who have undergone CT examination of the upper abdomen (3, 4). Because of the risk of malignancy, if the tumor is hormonally inactive, the question arises whether adrenalectomy should be performed or whether a conservative management, including clinical follow-up and serial CT scans, would suffice. Today, the decision is based mainly on the tumor size. Because malignant adrenocortical tumors have a poor prognosis, clinical trials using adjuvant therapy have been advocated (5). This strategy demands an accurate diagnosis. Today, it is often difficult to distinguish the malignant and benign tumors histopathologically. Markers to predict the natural course of adrenocortical tumors are urgently required, and could be based on the improved understanding of the molecular pathogenesis of the tumors.

The genetic aberrations involved in sporadic primary adrenocortical tumors are not completely understood, and little is known of their clinical impact. Previous studies have focused on selected chromosome regions. For example, Yano et al. (6) studied LOH in one primary and eight recurrent sporadic adrenocortical cancers as well as in eight sporadic benign lesions. The cancers showed LOH on 17p, 11p, and 13q. No genetic changes were found in any of the benign lesions. Although most of the adrenocortical tumors are apparently sporadic, these tumors are also found in conjunction with inherited cancer forms, such as the Beckwith-Wiedemann (11p15.5), Li-Fraumeni (p53 locus at 17p13), and multiple endocrine neoplasia type 1 (MEN1 at 11q13) syndromes. Genetic changes at these loci have been found in both hereditary and sporadic adrenocortical tumors (7-14). Karyotype analysis of two sporadic adrenocortical cancers have revealed alterations at 11p (15, 16).

CGH is a molecular cytogenetic technique which allows for the detection of DNA sequence copy number alterations across the entire genome in a single experiment. Regions with increased copy number reveal chromosomal sites that may contain dominant oncogenes, whereas regions with decreased copy number may harbor TSG loci. CGH has been used to detect LOH in tumors and normal tissues. The technique is based on the comparison of the DNA content of the tumor and a reference DNA sample, usually normal DNA from the same individual. A probe is used to detect the ratio of alleles at a specific locus, and this ratio is compared to the ratio of a reference probe. The ratio is calculated as the ratio of the fluorescence intensity at the locus of interest to the fluorescence intensity at a reference locus.

MATERIALS AND METHODS

This study included 22 primary sporadic adrenocortical tumors (8 malignant and 14 benign). All except two of the patients were operated on at the Karolinska Hospital between 1986 and 1993. Median follow-up time was 4.0 (range, 0.3-8.8) years. The tumors were classified as malignant or benign based on histopathological findings (invasion, necrosis, pleomorphism, and number of mitoses), tumor size (median, 4.0 cm; range, 0.5-20), steroid profile (19), and clinical outcome (Table 1).

DNA Preparation. High molecular weight DNA was isolated from fresh-frozen tumor tissues according to standard procedures. To ascertain the representativity of the material, sections were cut from all specimens for histopathological examination by one of the authors (A. H.). The fraction of tumor cell nuclei was estimated macroscopically. A limited number of samples that were classified as having 50-80% tumor cells were further investigated with classical point counting using a grid in the objective of the microscope. All samples were estimated to consist of >65% tumor cells (mean, 81%; range, 65-95%).

CGH. The CGH method has been described in detail elsewhere (17, 18). Four hundred ng of tumor DNA labeled by nick translation with a green fluorochrome FITC-dUTP, 400 ng of reference DNA labeled with Texas red-dUTP, and 10 mg of unlabeled Cot-1-blocking DNA were cohybridized to a fluorescein isothiocyanate (FITC)-labeled reference DNA and red-dUTP. The slides were washed and counterstained with DAPI in an antifade solution.

Digital Image Analysis. Evaluation of CGH results was performed using a digital image analysis system based on an Olympus BX50 fluorescence microscope and a CCD camera (Xillix, Inc., Vancouver, British Columbia, Canada) interfaced to a Sun workstation (Sun Microsystems Computer Corp., Mountain View, CA). For each hybridization, three single-color images were collected (matching DAPI, FITC, and Texas Red) from three to five metaphase spreads. The criteria for acceptable hybridization were uniform, strong hybrid-
Most Common Regions Involved in DNA Sequence Gains and Losses in Adrenocortical Cancers. The chromosomal regions with increased and decreased DNA sequence copy number are illustrated in Fig. 2. The most common regions of increased relative copy number were on chromosomes 4q (4/8, 50%), 5p (4/8, 50%), and 5q (4/8, 50%). The minimal common region of involvement was 4q31, whereas changes on chromosome 5 could not be further delineated. Chromosomes 12, 15q, 16q, and 19p also showed gains (three of eight). Minimal common regions were 12cen-q24, 15q2l-qter, 16q, and 19p.

DISCUSSION

This study represents the first genome-wide survey of DNA sequence copy number changes in adrenocortical tumors. A relationship between increasing number of genetic aberrations with increasing tumor size was obvious. All except 1 of the 8 cancers showed both gains and losses, whereas genetic aberrations were only found in 2 of the 14 adenomas. The most common genetic aberrations found were gains on chromosomes 4 and 5 as well as losses on chromosomes 2, 11, and 17. These chromosomes are likely to be important for adrenocortical tumorigenesis.

**RESULTS**

**Genetic Aberrations in Sporadic Adrenocortical Tumors Detected Using CGH.** Genetic aberrations were found in only 2 of 14 adenomas (Table 1). One of these showed gain of 9q as the only aberration, while the other showed gain of 1q as well as loss of 1p. In the remaining 12 adenomas (86%), no genetic aberrations were detected. In contrast, seven of the eight cancers showed both DNA gains and losses. The mean number of aberrations in the cancers was 10 (range, 0–25).

There was a strong relationship between tumor size and the number of genetic alterations. No alterations were seen in the smaller adenomas (<5 cm), whereas the two largest adenomas (5 cm each) both had genetic alterations. Among the eight cancers (7–20 cm), the number of genetic changes increased with larger tumor size (Fig. 1 and Table 1).

**INTERPRETATION OF CGH RESULTS.** An increase in the green:red ratio above 1.15 was considered to represent relative increases (gains) of DNA copy number in the tumor DNA relative to the average copy number in the normal reference genome. Similarly, a decrease in the ratio below 0.85 was considered as a relative loss of DNA sequence copy number. The cutoff values were derived from analyses of negative control experiments (included in each set of experiments) in which two differently labeled normal DNAs were simultaneously hybridized to the same metaphase. The heterochromatic regions (centromeres and telomeres) and the Y chromosome were not included in the interpretation due to the high degree of repetitive sequence. More details on the interpretation of CGH data have been published elsewhere (18).

**DNA Cytometry.** The DNA content of 8 cancers and 14 adenomas were analyzed using flow cytometric and image cytophotometric methods described previously (20).

### Table 1 Clinical and genetic data of patients with adrenocortical tumors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)/sex</th>
<th>Clinical appearance</th>
<th>Steroid profile in urine</th>
<th>Clinical outcome</th>
<th>Follow-up (yr)</th>
<th>Size (cm)</th>
<th>Histopathological diagnosis</th>
<th>DNA Cytometry</th>
<th>CGH analyses number of Gains</th>
<th>CGH analyses number of Losses</th>
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<td>1</td>
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<td>11</td>
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<td>2</td>
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<td>Rec, DFD</td>
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<td>A</td>
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<td>No rec, alive</td>
<td>7.5</td>
<td>9.0</td>
<td>Cancer</td>
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<tr>
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<td>Adenoma</td>
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<tr>
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<td>Adenoma</td>
<td>D</td>
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In urine

**DNA Cytometry.** The DNA content of 8 cancers and 14 adenomas were analyzed using flow cytometric and image cytophotometric methods described previously (20).

**Comparison among CGH, DNA Cytometry, and LOH Measurements.** All of the benign lesions showed a diploid DNA pattern using DNA cytometry, whereas all cancers were aneuploid. CGH analysis revealed DNA sequence alterations in all except one triploid cancer (Fig. 4). In an ongoing study, an allelotyping of 58 adrenocortical tumors was performed. The study includes the tumors in the present report and the correlation between the two methods was >90% based on 378 informative comparisons.

**REFERENCES**

CGH has been shown to be a powerful method for detecting genetic aberrations in a variety of solid tumors such as breast, prostate, and lung cancer (21–23). For example, in breast cancer, several novel sites of amplification have been observed (21). DNA sequence losses detected using CGH correlate well with studies of LOH/AI on the subchromosomal level (22, 24). Our allelotyping of adrenocortical tumors correlates >90% with the CGH data based on 378 informative comparisons. The limitation with the PCR-based detection of LOH/AI is that gains cannot be reliably distinguished from losses, and that interstitial and/or small deletions are missed if the marker is not located in the deleted region. On the other hand, CGH cannot reveal losses if the region affected is smaller than 10 Mb, if an allelic loss arises as a result of mitotic recombination, or if one allele is lost and the remaining one duplicated.

Six of the tumors were true incidentalomas, i.e., detected by using CT scan incidentally. When an incidentaloma has been found and metastasis from other primary sites and endocrine activity have been excluded, the question arises whether or not adrenalectomy should be performed due to the risk of malignancy. Today, the decision is based almost solely on tumor size, and surgery is recommended for tumors exceeding 3–6 cm (3, 25, 26). The CGH data showed that genetic changes were found only in tumors with a diameter of 5 cm or more, and that the number of alterations per tumor increased with increasing tumor size. This suggests that clonal proliferation and genetic instability often begin to emerge in tumors larger than 4 cm, and that this may be accompanied by malignant transformation. This idea is supported by previous studies that have shown adrenocortical cancers to be monoclonal, whereas adenomas may be either monoclonal or polyclonal, with a tendency for monoclonality in the larger adenomas (27).

Thus, the data on genetic changes support the clinical observation that a fundamental transition of growth characteristics and malignant progression occurs at a tumor size of 4 cm or more.
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behavior may take place when tumors become larger than 5 cm. Identification of the crucial genetic alterations will permit development of DNA-based diagnostic tools. Today, tumor size is used as the main preoperative indicator of malignant potential, but the addition of genetic markers could further help to characterize the tumor phenotype and thereby guide the treatment. Technically, these analyses can be performed on tumor material obtained by fine-needle aspiration or biopsy (28, 29). The present CGH findings provide starting points for refined mapping of TSG loci involved in this neoplasia. Several previously unreported losses were found, such as those involving chromosomes 2, 3p, 8p, 9p, 18q, and 22q. Whereas most of the aforementioned losses are common in other malignancies, chromosome 2 losses may be more specific to this neoplasia. The Carney complex, an autosomal dominant syndrome characterized by multiple neoplasias including adrenocortical tumors, has recently been mapped to 2p16 (30). Also, chromosome 2 harbors TSGs such as the two mismatch repair genes involved in hereditary nonpolyposis colon cancer, MSH2 and PMS1. On the other hand, our results are in agreement with previous studies of LOH/AI in recurrent sporadic adrenocortical tumors implicating chromosome 11p, 13q, and 17p as important chromosome regions for tumor progression (6). Moreover, we found relative losses of the 11q13 region (MEN1 locus) in three of eight sporadic cancers. However, CGH did not detect loss at the 11q13 region in any of the eight benign aldosterone-producing tumors as has been reported in previous LOH/AI studies (14). No high level amplifications were found in our material, indicating that activation of dominant oncogenes by this mechanism may not be common in adrenocortical cancers. Gain at chromosome 4 was found in four

Fig. 3. Green (tumor DNA):red (normal DNA) fluorescence intensities for chromosomes 2, 4, 5, 11, and 17 after CGH on tumor case 1 illustrated by digital images (a) and mean green:red ratio profiles (b). A decreased ratio is observed at chromosomes 2, 4cen-4q28, 11, and 17 and increased at chromosomes 4p, 4q31-qter, and 5. ---, ratios of 0.85 (bottom line) and 1.15 (top line). The chromosome diagrams above each ratio profile are shown for approximate visual comparison. The chromosomal localizations were done directly in relation to DAPI-banded metaphase chromosomes.
cancers with a minimal overlapping region at 4q31 suggesting the location of a putative overexpressed oncogene. In three tumors, the entire chromosome 5 was gained, possibly reflecting trisomy of this chromosome.

Overall, the CGH and DNA cytometry results reveal that sporadic adrenocortical cancers are genetically highly complex. An aneuploid DNA pattern was strongly associated with the presence of multiple genetic abnormalities detected using CGH. The cancer in which no changes were detected using CGH had a triploid DNA pattern. On the other hand, DNA cytometry was normal in two adenomas, whereas CGH was able to detect one and two changes, respectively.

Our CGH results implicate several previously unreported genetic changes that may have an important role in the development of sporadic adrenocortical cancers. The genetic changes may help to pinpoint locations of critical genes underlying development of this tumor type. Furthermore, the results indicate that the presence of genetic changes correlates strongly with tumor size and malignancy. The emerging pattern of genetic changes associated with tumor development and progression may turn out to be helpful in evaluating the malignant potential of adrenocortical tumors.

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Cancer Res 1996;56:4219-4223.