0021-972X/99/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 84, No. 3
Printed in U.S.A.

Comparative Genomic Hybridization Analysis of Adrenocortical Tumors of Childhood*

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ABSTRACT

Although several genes have been investigated in adrenal tumorigenesis, the genetic background of adrenocortical tumors (ACT) remains poorly characterized. In southern Brazil, the annual incidence of ACT is unusually high, ranging from 3.4–4.2/million children, compared with a worldwide incidence of 0.3/million children younger than 15 yr. Environmental factors have been implicated because the distribution of these tumors follows a regional, rather than a familial, pattern. However, decreased penetrance of a particular gene defect cannot be excluded. Because linkage or other traditional genetic analyses would not be appropriate to investigate the defect(s) associated with ACT in this population, we used comparative genomic hybridization (CGH) to screen for DNA sequence copy number changes in 9 nonfamilial ACT (6 carcinomas and 3 adenomas) from unrelated patients from this region. Six female (aged 10 months to 6 3/4 yr) and 3 male (1 1/12 to 3 1/4 yr) patients were studied. Three carcinomas were at stage I, 1 was at stage II, and another was at stage III. Two carcinomas had evidence of invasion of the vena cava, and 3 were more than 3 cm in size. All patients underwent surgical excision of their tumors; chemotherapy was administered to cancer patients. Currently, all patients are alive and in remission, with the exception of 1 patient with stage III cancer. High mol wt DNA was extracted from tumor tissue obtained at surgery and frozen at −70 C. This DNA was labeled and used for CGH according to standard procedures. Digital image analysis was performed to detect chromosomal gains or losses. CGH evaluation revealed extensive genetic aberrations in both adenomas and carcinomas; there were no significant differences relative to age, gender, size, or stage of the tumor ($P > 0.1$). Chromosomes and chromosomal regions 1q, 5p, 5q, 6p, 6q, 8p, 8q, 9q, 10p, 11q, 12q, 13q, 14q, 15q, 16, 18q, 19, and 20q demonstrated gains, whereas 2q, 3, 4, 9p, 11, 13q, 18, 20p, and Xq showed losses. The most striking finding was consistent copy number gain of chromosomal region 9q34 in 8 of the 9 tumors. We conclude that both benign and malignant ACT from southern Brazil show multiple genetic aberrations, including a consistent gain of chromosomal region 9q34. This genomic area may harbor genetic defects that predispose to ACT formation and are shared by the patients who were investigated in this study or are accumulated epigenetically under the influence of a common factor, such as an environmental mutagen. (*J Clin Endocrinol Metab* **84:** 1116–1121, 1999)

ALTHOUGH several genes (reviewed in Ref. 1) have been investigated in adrenal tumorigenesis, the genetic background of adrenocortical tumors (ACT) remains poorly characterized. Adrenocortical hyperplasia is a polyclonal process, but ACT are mostly monoclonal lesions (2), indicating that genetic changes at specific loci in the genome are needed for adrenal tumorigenesis. A number of chromosomal abnormalities have been implicated in this process, including genomic loci on 11p and 17p (3–6), which harbor genes with tumor suppression or oncogenic function in the adrenal cortex. These include the genes coding for the p53 (on 17p13.1) (7, 8) and p57 (on 11p15.5) (*KIP2*) (9, 10), and the insulin-like growth factor type II (on 11p15.5) (11–13) proteins.

However, none of these genes appears to be specific for ACT pathogenesis. Patients with Li-Fraumeni syndrome (LFS), who have germline p53 mutations (7), rarely develop adrenal cancer. An analysis of 475 tumors in 91 families with LFS revealed that breast cancer, bone and soft tissue sarcoma, and brain tumors are most frequent, whereas adrenal cancer developed in only 1% of the patients (14). In sporadic ACT, somatic p53 mutations may be present in approximately 30–50% of the malignant lesions (1, 15, 16). However, p53 expression does not correlate with prognosis, and it is rarely seen in sporadic, highly differentiated tumors, indicating that p53 mutations in malignant ACT are a late event in the process of tumorigenesis (17, 18). Thus, other genetic events precede and may even predispose to p53 mutations in ACT formation.

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that allows a genomewide screening of tumor DNA to identify chromosomal gains and losses (19,

Received July 20, 1998. Revision received November 23, 1998. Accepted December 10, 1998.

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* This work was supported in part by a CNPq grant (522775/96–9) and was presented (in part) in abstract form at the 80th Annual Meeting of The Endocrine Society, New Orleans, LA, June 21–27, 1998.

20). Regions of gains may contain dominantly acting oncogenes, whereas tumor suppressor genes may map to deleted regions (21). One important advantage of CGH is that frozen or paraffin-embedded samples can be evaluated because only tumor DNA, not cells in culture, is required for the analysis.

Other studies have shown genetic aberrations in ACTs from adult patients, but there is a paucity of information about these tumors in childhood. Kjellman *et al.* investigated by CGH the chromosomal imbalances of 8 carcinomas and 14 adenomas from adult patients. The most common genetic aberrations in carcinomas were gains of chromosomes 4 and 5 and losses of chromosomes 11 and 17 (22). However, many of the epidemiological and clinical features observed in children with ACT are different from those seen in ACT of adult patients, suggesting that childhood and adulthood ACT may be different disease entities and, perhaps, caused by different genetic processes (1). The great majority of the tumors in children cause signs and symptoms due to increased production of hormones, sex steroids, and/or glucocorticoids. By contrast, most of the tumors in adults do not cause Cushing's syndrome and/or virilization (23–26; reviewed in Refs. 27 and 28).

In the present study, we used CGH to investigate genetic events leading to ACTs in children from a region in southern Brazil (Curitiba), which, along with the state of Sao Paulo, has the highest incidence of these tumors worldwide (3.4–4.2/million children below 15 yr of age) (29). These patients do not have a diagnosis of any genetic syndrome; they are mostly female and under 4 yr of age, and their tumors have better prognosis than those of adults with comparable stages of tumor development (29). These features suggested that particular genetic events, germline or somatic, may be inducing tumorigenesis in this population. Indeed, CGH showed that 8 of the 9 tumors that were investigated exhibited, among other changes, copy number gain of a segment of chromosome 9 corresponding to cytogenetic band 9q34, suggesting that a gene(s) in this locus may play a role in the molecular process leading to ACT formation in this population.

Subjects and Methods

Patients

The adrenocortical tumors were diagnosed by criteria described previously (24, 29). Eight families were chosen for the present studies based on availability of patients for tumor sampling during surgery. All patients signed a written consent form for a research protocol approved by our hospital. All kindreds are genetically heterogeneous descendants of European origin (Italian, Polish, Portuguese, Spanish, or German) mixed with the local Indian population. There was no known consanguinity, and patients with known genetic conditions were excluded from this analysis.

All patients, six girls (aged 9–82 months) and three boys (aged 13–35 months), were referred to the Pediatric Endocrinology Unit of Hospital de Clínicas from Federal University of Paraná (Curitiba, Brazil). The profiles of all nine patients are given in Table 1. The clinical appearance was 1) virilization and Cushing's syndrome (n = 3), 2) only virilization (n = 5), and 3) only Cushing's syndrome (n = 1). The extent of the tumor by image analysis, the treatment regimens, and follow-up times are also given in Table 1. Steroidogenesis was evaluated by measurement of dehydroepiandrosterone, dehydroepiandrosterone sulfate, testosterone, 17-hydroxyprogesterone, and cortisol. Computed tomography scan of the abdomen and an angiographic exam of inferior vena cava (when computed tomography indicated invasion of the vena cava) were performed in all patients.

Tumors and DNA extraction

The tumors were classified as adenomas or carcinomas according to conventional pathological criteria, as previously described (29). After surgical excision, the tumor mass was sectioned, and the tumor fragments were frozen in liquid nitrogen. Before DNA extraction, the samples were examined by a pathologist, and only those specimens with pathological cells were used for DNA analysis. Thus, the vast majority of the cells of the eight primary tumors were tumor cells. One tumor (from patient 3) was a secondary local recurrent adrenocortical carcinoma, which was diagnosed almost 2 yr after resection of the primary mass. In this case, the neoplasm was highly vascularized, and a larger percentage of normal cells were present; the tissue was carefully resected for DNA extraction to avoid contamination by normal cells. DNA was extracted from all samples, as previously described (20, 30), and processed for CGH (20) in a way similar to that described for other ACT (22, 31).

CGH

CGH was performed according to standard procedures (20). Normal control DNA was prepared from peripheral blood lymphocytes of a cytogenetically normal male. Genomic DNA was extracted from the

TABLE 1. Clinical profile and management of patients with adrenocortical tumors investigated in this study

Patient no.	Gender	Age (months)	Imaging		Surgery	Tumor			Chemotherapy	Follow-up (months)
			CT	CAVA		Stage[a]	Vol (ml^3)	Pathology		
1	F	9.5	Nodular		TCE	I	27	CA		Well (15)
2	F	9	Nodular		TCE	I	25	CA		Well (41)
3[b]	F	82	Nodular	CIA	PR CI	III	650	CA	ICEM	D (19)
4	M	23	Nodular		TCE	I	25	CA		Well (24)
5	M	13	Nodular	CIA	TCE	II	380	CA		Well (7)
6	F	17	Nodular		TCE	I	26	CA		Well (17)
7	F	27	Nodular		TCE		48	Ad		Well (10)
8	M	35	Nodular		TCE		16	Ad		Well (14)
9	F	68	Nodular		TCE		5	Ad		Well (9)

F, Female; M, male; CT, computed tomographic scan of the adrenal glands; CAVA, inferior vena cava angiography; CI, inferior vena cava invasion as seen in CT; CIA, inferior vena cava invasion to the right atrium; nod, nodular appearance of the adrenal glands on CT; PR, partially removed; TCE, tumor completely excised; CA, carcinoma; Ad, adenoma; Chemo, chemotherapy; ICEM, isofosfamide, carboplatinum, etoposide, and mitotane; DS, deceased.

[a] Tumor staging as reported previously (1, 29).

[b] This patient died of complications of tracheostomy during his hospitalization in the intensive care unit.

frozen tissue using the Qiagen kit (Qiagen, Inc., Chatsworth, CA). Nick translation was performed to label tumor DNA with bio-16-deoxy-UTP (Boehringer Mannheim, Mannheim, Germany) and control DNA with digoxigenin-11-deoxy-UTP (Boehringer Mannheim). Five hundred nanograms of each of the labeled genomes were hybridized in the presence of excess Cot-1 DNA (50 181 g; Life Technologies, Gaithersburg, MD) to metaphase chromosomes prepared from a karyotypically normal donor. The biotin-labeled tumor genome was visualized with avidin conjugated to fluorescein isothiocyanate (Vector Laboratories, Inc., Burlingame, CA), and the digoxigenin-labelled control DNA was detected with a mouse antidigoxin antibody (Sigma Chemical Co., St. Louis, MO), followed by detection with a goat antimouse antibody conjugated to tetra-methyl-rhodamine isothiocyanate (Sigma Chemical Co.). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole and embedded in antifading agent to reduce photobleaching.

Microscopy and digital image analysis

Gray scale images of the fluorescein isothiocyanate-labeled tumor DNA, the tetra-methyl-rhodamine isothiocyanate-labeled control DNA, and the 4′,6-diamidino-2-phenylindole counterstain from at least eight metaphases from each hybridization were acquired with a cooled charge-coupled device CCD camera (CH250, Photometrics, Tucson, AZ) connected to a Leica Corp. (Deerfield, IL). DMRBE microscope equipped with fluorochrome specific optical filters TR1, TR2, and TR3 (Chroma Technology, Brattleboro, VT). Quantitative evaluation of the hybridization was performed using commercially available software (Applied Imaging, Pittsburgh, PA). Average ratio profiles were computed as the mean value of at least eight ratio images to identify chromosomal copy number changes in all cases.

Results

Clinical description

The clinical profile of the patients is summarized in Table 1. Six female (aged 10 months to 6 3/4 yr) and 3 male (1 1/12 to 3 1/4 yr) patients were studied. Clinical symptomatology differed in that none of the patients with adenomas (cases 7, 8, and 9) presented with signs of Cushing's syndrome; five of the six patients with carcinoma (cases 1–6) had Cushinoid features, although only two had consistently elevated urinary cortisol levels. Hirsutism and biochemical hyperandrogenism were shared by almost all patients regardless of the final pathological diagnosis. Carcinomas were diagnosed at stages I (3), II (1), and III (1). Two carcinomas had evidence of invasion of the inferior vena cava, and three were more than 3 cm in size. All patients underwent surgical excision of their tumors; chemotherapy was administered to one patient (case 3). Currently all patients are alive and in remission, with the exception of one patient with stage III cancer, who died in the intensive care unit as a result of complications of a tracheostomy.

CGH analysis

CGH was used to map DNA copy number aberrations that occur in these tumors; the DNA was extracted from frozen tumor samples. For patient 3, who was diagnosed with a stage III carcinoma, only metastatic tumor was available. Average ratio profiles were used for the mapping of copy number changes in all instances. A summary of all the changes is presented in Fig. 1, and an example of CGH analysis for chromosome 9 is presented for patients 5, 7, and 8 as an average ratio profile of 17, 20, and 21 metaphases, respectively, in Fig. 2. Chromosomal aberrations were detected in all 9 cases, and the average number of chromosomal aberrations per case was 8.2. There was no difference in the average number of changes seen in adenomas and carcinomas; there were also no significant differences with regards to age, gender, size, or stage of the tumor ($P > 0.1$).

Despite the high number of chromosomal aberrations, a recurrent pattern emerged. The most consistent finding was a gain of the long arm of chromosome 9 (or a portion of it), which was detectable in eight of the nine patients (89%). The smallest region of overlap that showed copy number gain was 9q34. A high level copy number increase (amplification) of 9q34 was observed in five of nine cases (56%). Figure 2 shows chromosome 9 profiles from three representative cases. In the ACT from patient 5 (*top panel*), chromosome 9 was normal; in the ACT from patient 8 (*middle panel*), CGH showed gain of 9q22qter and an amplification of 9q34; in the ACT from patient 7 (*lower panel*) CGH revealed amplification of just the chromosomal area corresponding to cytogenetic band 9q34.

The second most common chromosomal gain was the gain of the long arm of chromosome 12 (12q) or a portion of it (seven of the nine ACT, or 78%). In particular, the 12q23q24 region showed a gain in five of the nine ACT (56%). Other chromosomal areas that showed gain of genetic material were also identified on chromosomes 5 (four of the nine tumors, 44%), 19 (44%) and 20 (44%). Smaller regions that showed high level copy number increases (amplifications) were mapped to chromosomal bands 6p21, 9q34, 11q13, 12q11q22, 12q23q24, and 13q22qter.

Losses of genetic material were not as frequent as gains. The most common chromosomal copy number decrease were mapped to chromosome 4 [in four of the nine ACT it included the entire chromosome 4, and in one of the nine tumors it included only the long arm (4q)] and to chromosomal region 2q22q34 (four of the nine ACT). Chromosome 17 loss (the chromosome that harbors the p53 gene) was not seen in any of the tumors.

Discussion

ACT are often diagnosed on the basis of the clinical appearance, steroid testing, and imaging studies. In most children, the adrenocortical carcinoma is still in stage II of the disease (27–29) when medical help is sought. In our series of six carcinomas, four of them were diagnosed at stage I, and only 1 was diagnosed at stage III. In addition, the male to female ratio in our patients (who were below the age of 4 yr) was 2:1 [it is 4:1 for our entire population studied by Sandrini *et al.* (29)], whereas a ratio of 3:2 for this age group has been reported by others (24, 25). It is possible that these differences in clinical appearance may point to variable genetic background of these tumors, as has been suggested for the ACTs occurring in various age groups of children (29, 32).

Chromosomal imbalances may be associated with malignancy or, rarely, may indicate the genetic origin of the tumor (19, 20, 22, 31, 32). In the latter case we would expect to find it in both benign and malignant ACT, assuming that the carcinoma could arise from monoclonal expansion of an adenoma, as previously suggested (1). In total, 3 benign and 6 malignant ACT from our patients were examined by CGH. This analysis showed that 8 of these tumors (3 adenomas and 5 carcinomas) had copy number gains of the long arm of

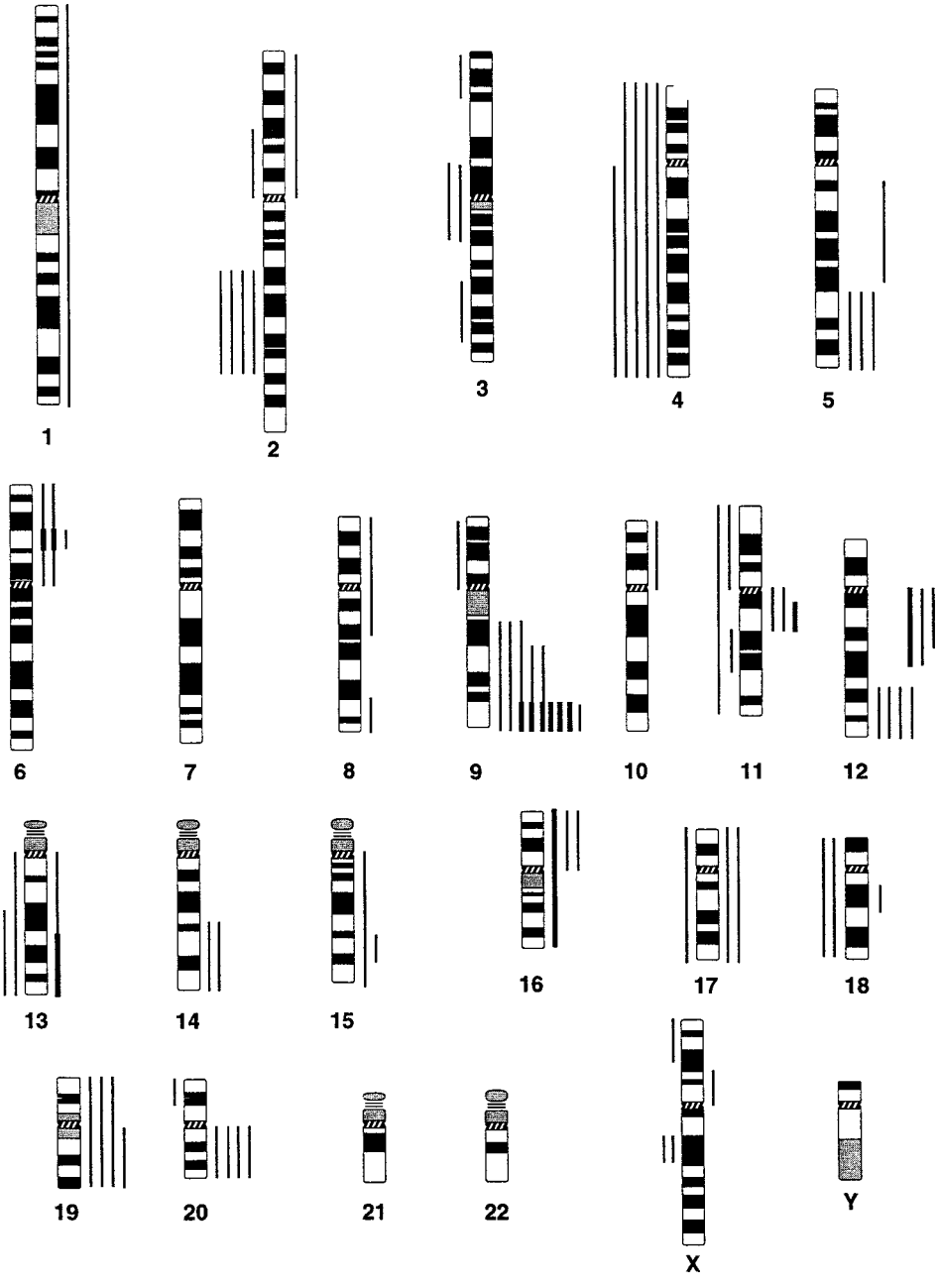

FIG. 1. Karyograms of chromosomal gains and losses in childhood adrenocortical tumors from southern Brazil (Curitiba). *Bars to the right* of the chromosome ideogram indicate a gain, whereas *bars to the left* indicate a loss of genetic material. *Bold lines* indicate amplifications.

chromosome 9. Region 9q34 was the smallest area of overlap and showed amplification in 5 tumors (4 carcinomas and 1 adenoma). Previous CGH survey of ACT genome from 22 adult patients found only 1 adenoma with gain of 9q (among 14 adenomas and 8 carcinomas) (22). This may indicate differences in the genetics of ACT formation between children and adults, which would be consistent with the epidemiological and other clinical differences in this disease in the 2 groups. Alternatively, 9q may only be involved in ACT formation in this population of children from Curitiba, southern Brazil. However, the fact that gain of 9q was seen in an adenoma in the study by Kjellman *et al.* (22), whereas it was seen in both adenomas and carcinomas in our study, indicates that this chromosomal region may indeed harbor a gene(s) important for early ACT formation in other patient groups as well.

One of the three most common regions of increased relative copy number reported by Kjellman *et al.* was 5q, which was found only in carcinomas (22). Indeed, gain of 5q31-qter (two of six carcinomas) and 5q14-q21 (one of six carcinomas) were also found in our study. The other two most common gains reported by Kjellman *et al.*, involving chromosomal areas 4p and 5p, were also found in one of the tumors of our study (a carcinoma).

Chromosomal gains were far more common than losses in our study. It is noteworthy that Reincke *et al.* also reported an excess of gains in their study of CGH of ACTs (31). As in our investigation, the chromosomal areas involved were the

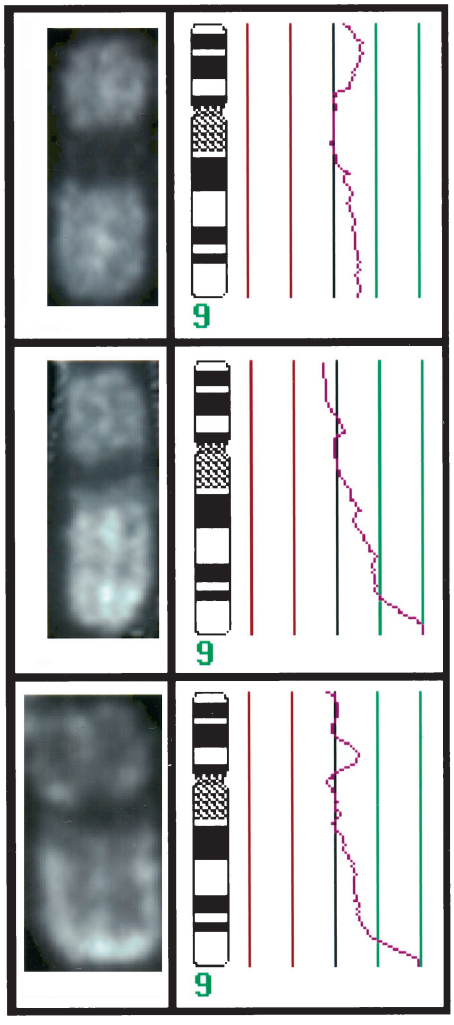

FIG. 2. Chromosome 9 CGH profiles from three representative cases. In case 5 (*top panel*), the profile was normal; in case 8 (*middle panel*), the profile showed a gain of 9q22qter and an amplification of 9q34; in case 7 (*bottom panel*), the profile showed an amplification of 9q34. The five *vertical lines on the right* of the chromosome ideograms reflect different values of the fluorescence ratio between the tumor DNA and the normal DNA. The values are 0.5, 0.75, 1, 1.25, and 1.5, from *left* to *right*. The *middle line* represents a 1:1 ratio and reflects a balance between the tumor DNA and the normal control DNA. The ratio profile (*curve*) was computed as a mean value of at least eight metaphase spreads. For each case, a representative chromosome 9 hybridized with tumor DNA is shown to the *left* of the ideogram. Notice that the regions of chromosomal gain correlate to an increased hybridization intensity. Chromosomal regions showing an amplification (9q34 for cases 8 and 7) show a further increase in the signal intensity compared to the rest of the chromosome.

same in adenomas and carcinomas. In our study, the most frequently lost chromosome region, found in four of our carcinomas, was deletion of 2q22q34. Kjellman *et al.* have also described loss in chromosome 2, although more common losses in their study were those involving chromosomes 11q and 17p (22). Although we also found a deletion of the area 11q14q22 in two carcinomas, we did not see loss of 17p in any of our tumors. This is particularly important, because chromosome 17 harbors the gene for p53 (7).

The p53 tumor suppressor gene is the most commonly altered gene in human cancers. Germline mutations in p53 are the genetic alteration underlying predisposition to multiple cancers in LFS. Most cases referred to the Hospital de Clinicas at Curitiba in southern Brazil (>85 tumors in the last 30 yr) are not associated with families presenting features of LFS. In the present series of affected children, 2 of them are relatives, but their families did not meet the diagnostic criteria of LFS. Furthermore, although our present study did not exclude the possibility of p53 mutations in these neoplasms, it is noteworthy that we did not find any loss of chromosome 17. A study by Ohgaki *et al.* found p53 mutations in only 3 of 15 adrenocortical carcinomas and 1 of 18 adenomas (18), suggesting that in sporadic ACT, p53 mutations are a late event in the multistep process of malignant transformation (1, 17). This is also supported by the finding of germline p53 mutations in 3 of 6 children with adrenocortical carcinoma (15).

It has been suggested that environmental mutagens may increase the rates at which cancer-predisposing somatic mutations occur (33). The role of agrotoxic compounds in the pathogenesis of ACT is controversial (29). The use of such compounds in agriculture in many areas of Brazil is arbitrary. As most ACT found in patients from southern Brazil and the state of Sao Paulo are sporadic, we have speculated that these compounds may play a role in the high incidence of ACT in these regions, especially as the incidence of other childhood tumors in these regions is not increased (29). We have been led to this speculation by the finding that mutations in the p53 and other genes are known to be selectively induced by environmental agents, such as aromatic amines, benzopyrene, and aflatoxin B (34–36). Although the present study was quite small and did not indicate that any tumor suppressor genes were of importance for ACT development in our patients, it is possible that environmental agents could cause additional "hits" required for adrenal tumorigenesis (6, 29), as predicted by Knudson's hypothesis (37). A larger study is required to examine this hypothesis and evaluate the consistency of losses of chromosomal material (indicating, perhaps, regions of loss of heterozygosity) that were found by CGH in our patients.

On the other hand, the finding of 9q34 amplification in our patients' tumors may indicate the site of the oncogene(s) important for ACT formation. Several candidate genes reside in this chromosomal region, which is also involved in the t(9;22) translocation seen in chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia (38, 39). In this process, the fusion of two genes, *BCR* and *ABL1*, gives rise to a BCR-ABL chimeric oncogene, which is generated by translocation of sequences from the c-ABL protein tyrosine kinase gene on 9q34 into the BCR gene in chromosome 22. In the pathogenesis of ACT, amplification of 9q34 could involve aberrant transcripts of the *ABL1* oncogene. Other potential candidate genes mapped to 9q34 (40) include the *vav2* oncogene (41), the transforming growth factor-β receptor-1 gene (*TGFBR1*), which is an activin A receptor type II-like kinase (42), the tumor necrosis factor receptor-associated factor-2 gene (*TRAF1*) (43), and the oncogene 24p3 or lipocalin 2 gene (*LCN2/NGAL*) (44).

In conclusion, we found that benign and malignant ACT from southern Brazil show multiple genetic aberrations and

a consistent gain and, in some cases, amplification of chromosomal region 9q34. These changes may reflect genetic defects that are shared by our patients and predispose to ACT formation or are accumulated epigenetically by a common factor regardless of genetic background. Further studies are required to characterize these chromosomal imbalances, especially the gain in 9q34, and to determine to what extent they are linked to the malignancy or etiology of these tumors.

Acknowledgments

We thank Dr. George P. Chrousos (Developmental Endocrinology Branch, NICHD, NIH) for his suggestions and support of this study, and Dr. Lawrence S. Kirschner (Unit on Genetics and Endocrinology, Developmental Endocrinology Branch, NICHD, NIH) for his helpful comments and assistance with some of the experiments. We also thank Ms. Martha Hunt (Georgetown University, Washington DC) for her technical assistance. Finally, we thank the patients and their families for providing tissue for these studies and wholeheartedly supporting our department in the clinical investigation of adrenal tumors in South Brazil.

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