



Comparative Genomic Hybridization Analysis of Human Parathyroid Tumors

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ABSTRACT: Primary hyperparathyroidism is characterized by hypercalcemia and elevated parathyroid hormone levels. It can be caused by overactivity of one (adenoma or carcinoma) or more (hyperplasia or multiple adenoma) parathyroid glands. Parathyroid adenoma and hyperplasia are usually mono- or oligoclonal neoplasms. To establish whether parathyroid cancer has a genetic composition distinct from parathyroid adenoma, we analyzed 10 adenoma and 10 carcinoma cases by comparative genomic hybridization (CGH). Results show clear differences between the constitution of adenoma and carcinoma genomic DNA. The most frequent genomic alterations in adenoma included deletions on chromosomes 11, 17 (5 of 10 cases), and 22 (7 of 10 cases). In parathyroid carcinoma, frequent chromosomal deletions were on chromosome arm 1p (4 of 10 cases) and chromosome 17 (3 of 10 cases), and gains were on chromosome 5 (3 of 10 cases). Our data indicate that different genetic changes could contribute to the development of parathyroid adenoma and carcinoma; genomic losses predominate in adenoma, and gains along with some losses are found in carcinoma. Furthermore, the CGH results implicate several chromosomal regions that may harbor genes that could be potentially involved in the development of parathyroid adenoma and carcinoma. Published by Elsevier Science Inc.

INTRODUCTION

Primary hyperparathyroidism (pHPT) is a common endocrine disorder characterized by dysregulation and excessive secretion of parathyroid hormone (PTH) from one or more parathyroid glands. PTH excess may be produced by a parathyroid adenoma, parathyroid hyperplasia, or a parathyroid carcinoma. The chief biochemical manifestation of primary PTH excess is hypercalcemia. Approximately 100,000 new cases of pHPT per year are estimated in the United States [1]. Most patients with pHPT (80–85%) harbor a single adenoma; the other three glands are normal. About 15–20% of the patients with pHPT have hyperplasia that involves enlargement of all four parathyroid glands occurring sporadically or in conjunction with an inherited disorder such as multiple endocrine neoplasia

(MEN) type 1 or 2. The rarest form of pHPT is parathyroid carcinoma with a single gland enlarged; this form is seen in well under 1% of all patients with pHPT [2]. The parathyroid adenoma is a solitary hyperfunctional benign monoclonal parathyroid tumor [1]. The carcinoma shows unrestrained growth, is often palpable, and usually presents with severe hypercalcemia. Additionally, the carcinoma tends to be locally invasive and has the ability to metastasize. Not enough evidence exists to show that parathyroid cancer usually arises from an adenoma.

Tumor cytogenetic analysis has so far not indicated the involvement of chromosomal target regions that could be intensively searched for direct-acting oncogenes or tumor-suppressor genes causing parathyroid lesions. A translocation between chromosomes 1 and 5 has been reported in a single parathyroid adenoma [3], but it is unclear whether this was an isolated random occurrence or will be characteristic of a distinct subset of adenomas. The cyclin *D1* (*PRAD1*) oncogene is rearranged with the PTH gene and is thereby transcriptionally activated in a very small subset of parathyroid adenoma [4, 5]. Loss of heterozygosity (LOH) analyses in parathyroid adenoma have detected allelic loss of chromosome 11q in 35–40%, LOH on chromosome arms 1p, 6q, 11p, and 15q in 30%, and allelic loss of 3q markers in 10% of cases [6–11]. Allelic loss of the retinoblastoma tumor-suppressor gene reported to be highly specific for parathyroid carcinoma [12, 13] also has been

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detected in 16% of parathyroid adenomas associated with aggressive clinical and histopathological features [14]. Allelic loss of the *p53* tumor-suppressor gene was observed in parathyroid carcinoma from 2 of 6 genetically informative patients and not in parathyroid adenoma from 20 informative patients [15]. Analysis of gene amplification has not been reported in parathyroid cancer or adenoma. Analysis of both gains and losses of chromosomal regions is possible with comparative genomic hybridization (CGH), first described by Kallioniemi et al. [16]. We have used CGH to screen 10 frozen parathyroid adenoma samples, 1 frozen parathyroid carcinoma, and 9 paraffin-embedded parathyroid carcinoma samples.

MATERIALS AND METHODS

Tissue Samples

Parathyroid adenoma samples were obtained from patients undergoing parathyroidectomy for the management of primary hyperparathyroidism at NIH. Patients exhibited accepted clinical features of sporadic parathyroid adenoma. No patient had MEN1 or a history of neck irradiation. All were hypercalcemic with elevated levels of parathyroid hormone. None of the adenomas had clinicopathological features of malignancy. After surgical removal, tissue was immediately frozen in liquid nitrogen and then stored at -70°C until DNA extraction.

Nine samples that were classified as parathyroid cancer from the AFIP registry were provided by Dr. Clara S. Heffess. These samples were obtained as archived blocks of formalin-fixed paraffin-embedded parathyroid tissues. Sections ($50\ \mu\text{m}$) of tissues from the blocks were cut and stored in microfuge tubes for DNA extraction. One frozen parathyroid cancer specimen was obtained from NIH.

All NIH patients gave informed consent according to the protocol approved by the NIDDK Institutional Review Board.

DNA Extraction

High-molecular-weight genomic DNA from parathyroid adenoma was extracted by using Trizol reagent from GIBCO/BRL (Gaithersburg, MD).

DNA from the $50\ \mu\text{m}$ sections of parathyroid carcinoma specimens was extracted as per the protocol described by Ried et al. [17]. Briefly, the sections were deparaffinized by washing twice at room temperature with xylene followed by 100% ethanol and dried under vacuum. The samples were treated overnight with 1 M NaSCN at 37°C . The tissue was washed and resuspended in $500\ \mu\text{L}$ of DNA isolation buffer containing 75 mM NaCl, 25 mM EDTA, and 0.5% Tween. Proteinase K was added to a final concentration of 1 mg/mL, and the tissue was incubated overnight at 55°C . The DNA was purified by phenol-chloroform extraction and ethanol precipitation. The molecular weight and purity of DNA were checked by agarose gel electrophoresis. Genomic DNA of molecular size more than 5 kb was considered suitable for CGH analysis. Normal control DNA for CGH analysis was extracted from blood of a healthy male donor by using standard methods [18].

Metaphase Chromosome Preparation

Metaphase chromosome spreads suitable for CGH analysis were prepared from peripheral blood lymphocytes of a normal healthy male by following standard procedures [19]. The preparations were chosen for CGH as per the criteria defined by Du Manoir et al. [20].

Labeling and Fluorescence In Situ Hybridization

Normal male DNA was labeled with digoxigenin-11-dUTP in a standard nick translation reaction (Boehringer Mannheim, Indianapolis, IN). Similarly, tumor DNA was labeled with biotin-16-dUTP. DNaseI concentrations in the reactions was adjusted to obtain a final average nick translated DNA fragment size of 500–1000 bp.

For CGH, 200 ng of digoxigenin-labeled normal DNA and 200 ng of biotin-labeled tumor DNA were ethanol precipitated in the presence of $10\ \mu\text{g}$ of salmon sperm DNA and $30\ \mu\text{g}$ of Cot-I fraction of human DNA (GIBCO/BRL). The probe mixture was resuspended in $10\ \mu\text{L}$ of hybridization buffer containing 50% formamide, $2 \times \text{SSC}$, and 10% dextran sulfate. Probe DNA was denatured at 80°C for 5 minutes and allowed to preanneal at 37°C for 2 hours. Metaphase spreads were pretreated with RNase as described [21] and dehydrated through an ethanol series. Pretreated metaphase spreads were denatured at 80°C for 2 minutes in 70% deionized formamide and $2 \times \text{SSC}$ and dehydrated through an ethanol series. Preannealed probe mixture was applied to the slide under a cover slip ($18\ \text{mm}^2$), sealed with rubber cement and hybridized at 37°C for 4 days.

Posthybridization steps were performed as described in detail by Ried et al. [21]. The hybridized biotinylated sequences were detected with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector, Burlingame, CA), and digoxigenin-labeled probe sequences were detected by tetraethylrhodamine isothiocyanate (TRITC)-conjugated anti-digoxigenin. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in an anti-fade agent (DABCO) to reduce photobleaching.

Microscopy and Digital Image Analysis

Gray level images of metaphase chromosomes were acquired for each fluorochrome with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) connected to a Leica DMRBE epi-fluorescence microscope. Chromosome identification was based on DAPI banding. Fluorescence ratios of images and the ratio profiles of individual chromosomes were determined by a custom computer program [20] run on a Macintosh Quadra 950. Briefly, after determination of the chromosomal axis of each chromosome in every metaphase, individual FITC and TRITC profiles were calculated. These profiles were used for the computation of FITC:TRITC ratio profiles (Fig. 1F). The three vertical lines on the right side of the chromosome ideogram represent different values of the fluorescence ratios of tumor versus control DNA. The values are 0.75, 1.0, and 1.25 from left to right. These values are chosen as thresholds for the identification of DNA copy number decreases (0.75) and increases (1.25) as described in [20]. For each case, the average profile was computed from at least

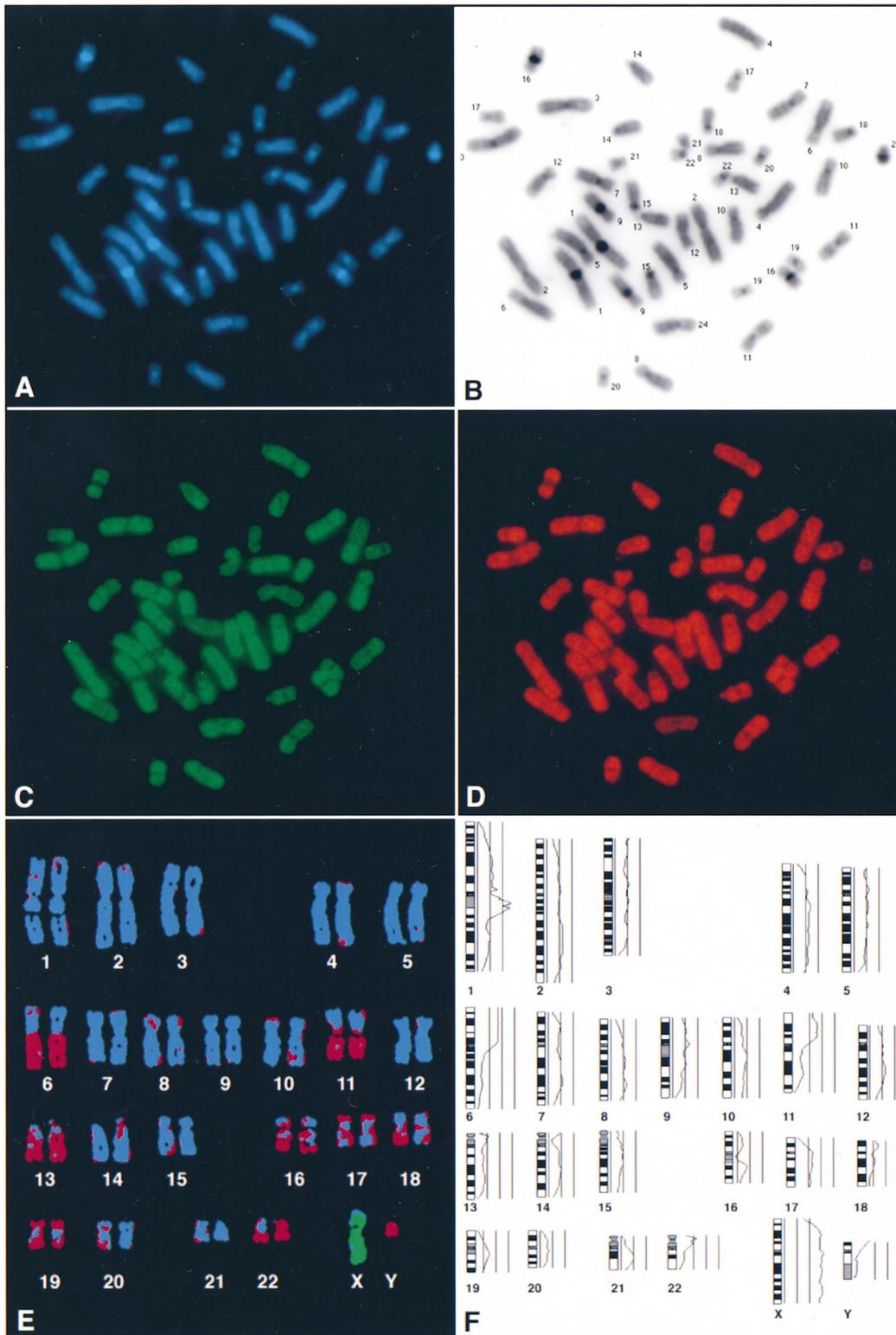


Figure 1 Complete CGH analysis of parathyroid adenoma case 6. DAPI (A) and inverted DAPI (B) images used for chromosome identification. FITC (C) and TRITC (D) images of tumor and normal control DNA hybridized to a metaphase spread. (E) Pseudocolor representation of fluorescence ratios: blue color depicts equal representation of tumor and control DNA on the chromosome, red shows DNA sequences that are underrepresented in the tumor, and green shows DNA sequences that are overrepresented in the tumor. (F) The average ratio profile of this tumor.

six metaphase spreads (at least 10 chromosomes of each type).

RESULTS

We used CGH to identify DNA sequence copy number changes in 10 parathyroid adenomas and 10 parathyroid carcinomas.

Parathyroid Adenoma

A complete CGH analysis of one parathyroid adenoma (case no. 6) is presented in figure 1. Figure 1C and D shows the FITC and TRITC images of tumor and control DNA hybridized to normal metaphase chromosomes. Figure 1A and B shows the DAPI and inverted DAPI images used for chromosome identification. Figure 1E shows the image of green-to-red fluorescence ratios of tumor versus control DNA presented in pseudocolor. Blue color depicts equal representation of tumor and control DNA on the chromosome, red shows DNA sequences that are under-represented in the tumor, and green shows DNA sequences that are overrepresented in the tumor. The X chromosome appears green because tumor DNA from a female patient was hybridized to male chromosomes. A compilation of fluorescence ratio profiles from seven different metaphases was used to calculate the average ratio profile of this patient and is shown in Figure 1F. Examples of copy number losses seen in this tumor are on chromosomes 13, 17, 18, 19, and 22 and chromosomal arms 6q, and 11q.

Figure 2 shows the summary of losses and gains of chromosome regions detected in 10 parathyroid adenomas. Of the 10 cases of parathyroid adenoma, one case (5) did not show any aberration. The most common losses in order of frequency were seen on chromosome 22 (7 of 10 cases), chromosomes 11 and 17 (5 of 10 cases), chromosome 19 (4 of 10 cases), chromosomes 3, 6, 13, 15, and 18 (3 of 10 cases), and chromosomes 9 and 16 (2 of 10 cases). Losses on chromosomes 4, 10, 12, 14, 20, and Y and chromosomal arm 1p were seen in single cases. Gains were seen only in single cases on chromosomes 2, 6p11–23, 6q11–23, 7, 11, and 21 and chromosomal arms 4p, 5q, 9p, and 20p.

Parathyroid Carcinoma

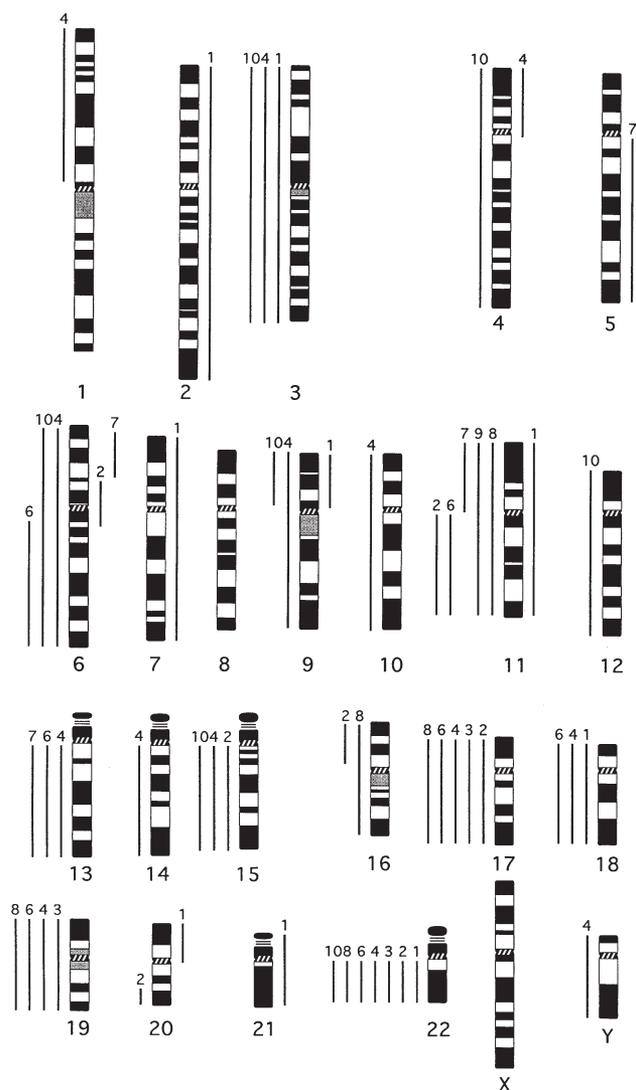
Figure 3 shows the summary of DNA copy number changes in 10 parathyroid carcinoma cases. Nine of these cases were archived formalin fixed paraffin embedded, whereas one sample (case 6) was a frozen specimen. Of the 10 cases of parathyroid carcinoma analyzed, 2 cases (cases 4 and 10) did not reveal any aberration. Most frequent losses were on chromosome arm 1p (4 of 10 cases), chromosome 17 (3 of 10 cases), and chromosomes 19 and 22 (2 of 10 cases). Losses on chromosomes 3, 6q11–qter, 7q11–qter, 8, 11q23–33, 13, 15, and 18 and chromosome arm 12p were seen in single cases. Gains were seen most frequently on chromosome 5 (3 of 10 cases), chromosomes 4, 12, and 16 and chromosome arms 1q, and 8q (2 of 10 cases). Gain of DNA sequences were also in isolated cases on chromosome arm 6q, and chromosomes 11q11–23 and 20.

A high-level increase in copy number (increased fluorescence ratios above the arbitrarily defined threshold value) of the entire chromosomes 4, 5, and 14 were seen in carcinoma case 3.

DISCUSSION

We performed CGH analysis of single gland parathyroid tumors, adenoma and parathyroid carcinoma, in an effort to scan the entire tumor genome for DNA sequence copy number changes. CGH has the power to perform a genome-wide analysis of both loss and gain of chromosomal regions that may harbor genes involved in the pathogenesis of different tumors. Not much data are available for parathyroid carcinoma owing to the lack of sufficient amount

Figure 2 Summary of chromosomal losses and gains in 10 parathyroid adenomas. Regions of loss are represented by lines at the left of the chromosomes; regions of gain are shown by lines at the right.



and preservation of tissue. CGH analysis is possible in the presence of 30–40% admixture of normal DNA and even with paraffin-embedded material [22]. In fact, we used archived formalin-fixed paraffin-embedded material (9 of 10 cases analyzed) for CGH from parathyroid carcinoma. A good agreement between data obtained from formalin-fixed paraffin-embedded and fresh-frozen specimens by using CGH was previously established by this group [17, 22] and others [23–25].

The pattern of chromosomal losses and gains appears to be complex for the adenomas, involving virtually every chromosome except chromosome 8, whereas, in the carcinomas, chromosomes 2, 9, 10, and 21 did not show any changes.

Allelic loss on chromosome arm 1p has been implicated in 30% of adenomas by LOH analysis [6]. In this study, CGH detected underrepresentation of DNA on chromosome arm 1p in 40% of carcinomas and in only 10% of adenomas analyzed. The region of loss on chromosome arm 1p in parathyroid adenomas may be below the limits of CGH resolution (10 Mb) and therefore not detected very frequently in the present investigation. Additionally, CGH cannot reveal an allelic loss resulting from mitotic recombination or if one allele is lost and the remaining one duplicated [26].

Two cases of parathyroid adenoma (cases 8 and 9) had loss of entire chromosome 11, and two cases (cases 2 and 6) had loss of chromosomal arm 11q. This finding is in agreement with the 35–40% LOH reported by several investigators for this region in sporadic parathyroid adenoma [7, 8, 10]. The MEN1 gene is one tumor-suppressor gene at 11q13 and has been shown to be mutated in at least 54% of sporadic parathyroid adenomas that possess LOH at 11q13 [27].

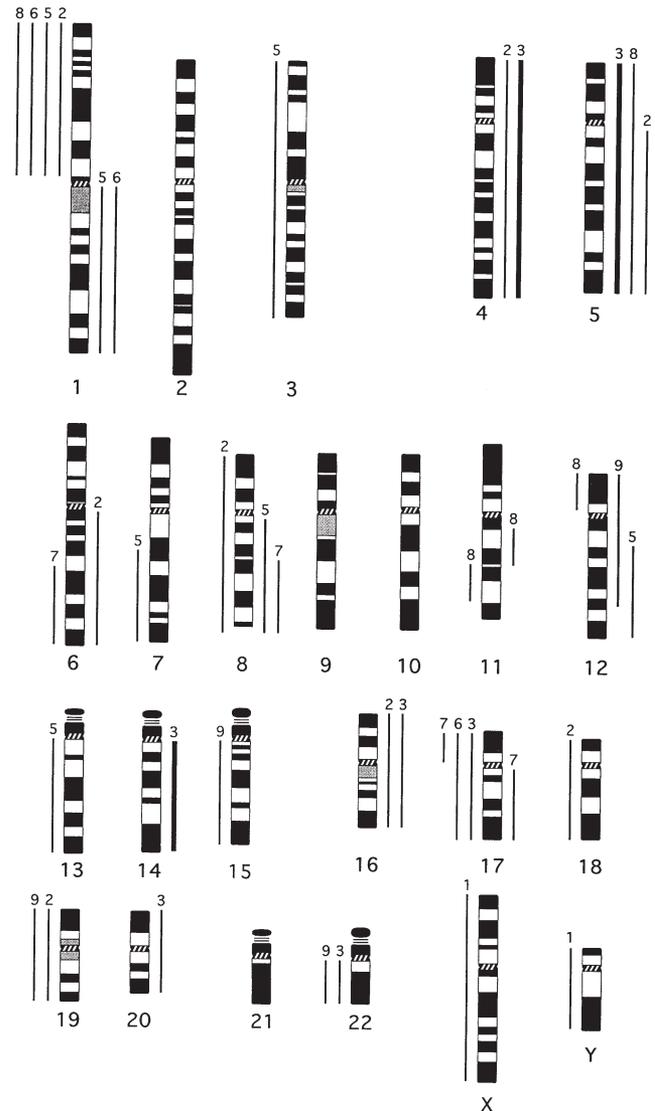
A striking finding is the underrepresentation of chromosomes 17 and 22, seen respectively in 5 and 7 of the 10 adenoma cases analyzed. Chromosome 17 loss can be associated with the loss of tumor-suppressor gene *p53*, which is lost in a large percentage of various cancers [28]. Loss of chromosome 22 is also seen in 35% of primary breast tumors [17] and meningiomas [29]. Although some comprehensive data for LOH analysis exist for parathyroid adenoma, several regions of losses were observed by CGH that are previously unreported for parathyroid adenoma, such as those involving chromosomes 17, 18, 19, and 22. Losses on these chromosomes have been reported in several malignancies [30], albeit most benign tumors do not exhibit many chromosomal aberrations [31].

Gains on chromosome 5, 4, and 16, and chromosome arms 8q and 1q seen in our carcinoma samples, which may contain dominant oncogenes, are also observed in other cancers. Frequent overrepresentation of chromosome arm 5p is reported for small-cell lung cancers [32, 33], cervical carcinomas [34], and head and neck squamous cell carcinomas [35], whereas gain of 5q is seen in renal cell carcinoma [36]. Gains on chromosomes 4 and 5 are also seen frequently in adrenocortical tumors [37]. DNA sequence copy number increases on chromosome arms 8q ad 16q are common in prostate cancer [26, 38, 39]; 8q and 1q in primary breast tumors [17, 40].

Our results show loss of DNA from the short arm of chromosome 17 in both parathyroid adenoma and parathyroid carcinoma (more frequently in adenoma), and the possibility of its involvement in the etiology of both tumor types cannot be ruled out.

The number of nonrandom chromosomal aberrations has been shown to increase significantly in the progression from low- and high-grade precursor lesions to invasive carcinomas in colorectal [41] and cervical tumors [42]. In this study, the average number of aberrations observed in parathyroid adenoma was 5.7 and in carcinoma was 4. This suggests that parathyroid adenoma is not likely to be a precursor of carcinoma and the two events

Figure 3 Summary of chromosomal losses and gains in 10 parathyroid carcinomas. Regions of loss are represented by lines at the left of the chromosomes; regions of gain are shown by lines at the right. High-level chromosome/region copy number increase (increased fluorescence ratios above the arbitrarily defined threshold value) is shown by thick lines.



could occur independently. Further analysis of a large number of parathyroid tumor specimens will be required to confirm this initial observation.

Our data indicate that different genetic changes could contribute to the development of parathyroid adenoma and carcinoma; losses predominate in adenoma and gains along with some losses define carcinoma. The zones of losses differ in adenoma and carcinoma. Furthermore, the CGH results implicate several chromosomal regions (some previously recognized and others newly identified) that may contain genes with an important role in the pathogenesis of parathyroid adenoma and carcinoma.

ADDENDUM

During the review process of the present investigation, a study describing CGH analysis of parathyroid adenomas was also published in *J Clin Endocrinol Metab* 83:1766–1770 (1998).

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