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From Amplification to Gene in Thyroid Cancer: A High-Resolution Mapped Bacterial-Artificial-Chromosome Resource for Cancer Chromosome Aberrations Guides Gene Discovery after Comparative Genome Hybridization

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Summary

Chromosome rearrangements associated with neoplasms provide a rich resource for definition of the pathways of tumorigenesis. The power of comparative genome hybridization (CGH) to identify novel genes depends on the existence of suitable markers, which are lacking throughout most of the genome. We now report a general approach that translates CGH data into higher-resolution genomic-clone data that are then used to define the genes located in aneuploid regions. We used CGH to study 33 thyroid-tumor DNAs and two tumor-cell-line DNAs. The results revealed amplifications of chromosome band 2p21, with less-intense amplification on 2p13, 19q13.1, and 1p36 and with least-intense amplification on 1p34, 1q42, 5q31, 5q33-34, 9q32-34, and 14q32. To define the 2p21 region amplified, a dense array of 373 FISH-mapped chromosome 2 bacterial artificial chromosomes (BACs) was constructed, and 87 of these were hybridized to a tumor-cell line. Four BACs carried genomic DNA that was amplified in these cells. The maximum amplified region was narrowed to 3–6 Mb by multicolor FISH with the flanking BACs, and the minimum amplicon size was defined by a contig of 420 kb. Sequence analysis of the amplified BAC 1D9 revealed a fragment of the gene, encoding protein kinase C epsilon (PKCε), that was then shown to be amplified and rearranged in tumor cells. In summary, CGH combined with a dense mapped resource of BACs and large-scale sequencing has led directly to the definition of PKCε as a previously unmapped candidate gene involved in thyroid tumorigenesis.

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Introduction

The association of specific chromosome abnormalities with particular types of human cancer has been established by investigators during the past decade (Mitelman 1994; Kallioniemi 1997). Such abnormalities can be characterized as changes in gene copy number that are due to either amplifications or deletion, such as those encoding oncogenes or tumor-suppressor genes, respectively (Gordon et al. 1994), or as translocations and other rearrangements, long known to be an important feature of certain hematologic malignancies (Brodeur et al. 1984; Bishop 1987; Slamon et al. 1989; Oliner et al. 1992).

Thyroid neoplasms represent an attractive model for the study of the molecular genetics of tumorigenesis, since they constitute a broad spectrum of phenotypes, ranging from benign follicular adenomas to the uniformly fatal anaplastic thyroid carcinomas. Current genetic studies indicate that the development and progression of thyroid tumors is signaled by phenotype-specific mutations of genes involved in growth control (Fagin et al. 1994).

Approximately 10% of the population develops palpable thyroid nodules during their lifetime. More than 12,000 new cases of thyroid cancer develop each year in the United States, for which scant information on cytogenetic abnormalities is available. Although, during the past few years, significant progress has been made in the identification of somatic-gene defects associated with various human thyroid-tumor phenotypes (Fagin et al. 1993), the diagnostic tools available to distinguish benign from malignant neoplasms are not reliable, resulting in a large number of unnecessary surgeries. Point mutations of ras oncogenes are believed to be important initiating events in thyroid-tumor formation (Lemoine et al. 1989; Namba et al. 1990; Suarez et al. 1990), and mutational activation of ras has been shown to promote genomic instability and gene amplification (Finney and

Bishop 1993; Denko et al. 1994). However, screening studies for potentially amplified regions in thyroid tumors have relied on candidate-gene markers and have been mostly unrewarding (Namba et al. 1990).

CGH is a recently described molecular-cytogenetic technology that globally assays for chromosomal gains and losses in solid tumors when chromosome preparations are not feasible (Kallioniemi et al. 1992, 1994*b*). Application of CGH to DNAs extracted from tumor specimens has revealed a number of chromosomal aberrations and imbalances that were undetected by traditional cytogenetic analysis. These findings warrant molecular-genetic studies aimed at isolation of the perturbed genes (Houldsworth and Chaganti 1994; Kallioniemi et al. 1994*a;* Ried et al. 1995, 1996; Voorter et al. 1995; Schrock et al. 1996). CGH has been used also to identify tumor-specific genetic markers and to evaluate the role of known genes in tumor diagnosis, prognosis, and progression (Forus et al. 1995; Visakorpi et al. 1995; Boerman et al. 1996; Ghazvini et al. 1996; Koivisto et al. 1997). In order to define candidate genes or regions involved in the chromosomal imbalance, FISH analyses have been performed on tumor samples (Tanner et al. 1994, 1996; Jossart et al. 1995, 1996; Taniwaki et al. 1995). However, despite the power of CGH for whole-genome analyses, no previously unmapped novel tumor gene has been identified by this approach. This limited ability to rapidly translate the global CGH approach into the identification of candidate genes has been hampered both by the low resolution of regional assignment of amplification and by the lack of large arrays of stable genomic clones located in the unbalanced regions.

To remedy this, we report an approach to tumor-gene discovery, after CGH, that combines the construction and application of a chromosome 2 high-density resource of mapped bacterial artificial chromosomes (BACs) (Chen et al. 1996) and large-scale sequencing, to investigate amplified sequences. By this approach, PKCε (the gene for protein kinase C epsilon) has been identified as a candidate for thyroid tumorigenesis (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data).

Material and Methods

Cell- and Tissue-Sample Collection

Human thyroid tumors were collected at the time of surgery and immediately were frozen in liquid nitrogen. Whenever possible, samples were taken from both the tumor and the normal tissue from the same individual. Histopathologic diagnosis was confirmed independently by at least two pathologists. Tissues used for the current study are listed in the Appendix. Samples for DNA extraction were taken from within the tumor nodules, at sites distant from the margins of the lesion. Representative sections were examined to ensure that the samples did not include normal thyroid or non-neoplastic tissue. Papillary carcinomas contain a variable admixture of stromal cells, but usually $\geqslant 70\%$ were tumor cells. The human thyroid carcinoma cell lines ARO and WRO were originally obtained from G. Juilliard (UCLA) and were propagated in RPMI-1640 medium containing 10% FCS, glutamine (286 mg/liter), and Fungi-Bact (Irvine Scientific, CA), as described by Zeki et al. (1994).

DNA Extraction and Southern Blot Analysis

DNA from snap-frozen thyroid tissue was prepared by use of the guanadinium-CsCl procedure (Davis et al. 1986). Southern blot analyses were performed as described elsewhere (Southern 1975). In brief, 10 mg of *Eco*RI-digested DNA was electrophoresed on a 0.9% agarose gel and were transferred to a nylon membrane. Membranes were hybridized with the indicated [^{32}P] random-primed probe, as described by the manufacturer (Strategene). After being washed at high stringency, blots were exposed to XAR-5 or Kodak BMR-1 films (Eastman Kodak).

CGH to Determine Chromosomal Regions Potentially Amplified in the Tumor

The CGH procedure was modified from that of Kallioniemi et al. (1992). To increase the resolution of CGH and to facilitate the subsequent assignment of mapped BACs to putatively amplified regions, the present study replaced the standard DAPI counterstain with a higher-resolution reverse-banding technique (Korenberg and Chen 1995). A total of 33 tumors and two cell lines were analyzed by CGH (see the Appendix). For each, the hybridization signal from the tumor or cell-line DNA was compared with germ-line DNA from either normal thyroid tissue or peripheral blood of the corresponding individual. When matched samples were not available, sex-matched control DNAs were used. To evaluate for abnormal ratios due to target structure or probe composition, control experiments were performed first, with normal genomic DNAs used as the competing probes. DNA labeling, hybridization, and detection were performed as described by Kallioniemi et al. (1992).

High-Resolution Banding and Image Acquisition for CGH

High-resolution chromosome identification was achieved by staining of slides, at room temperature, with chromomycin A3 (0.3 mg/ml in 1/2x McILavane's buffer pH 9.0) for 15 min, followed by staining with distamycin A (0.1 mg/ml in 1/2 McILavane's buffer, pH 9.0) for 1 min, a modified reverse-banding technique described

Table 1

Aneuploid Regions in Thyroid-Tumor and Cell-Line DNAs

Aneuploid Region	No. of Cases							Positive DNA (%)
	Follicular Adenoma ($n = 10$)	Follicular Carcinoma		Papillary Carcinoma ($n = 16$)	Anaplastic Tumor		Total ($n = 32$)	
		Tissue ($n = 4$)	Cell Line ($n = 1$)		Tissue ($n = 1$)	Cell Line ($n = 1$)		
Amplified (green/red > 1.5):								
1p34	2	2	…	4	…	…	9	28
1p36	3	2	…	5	…	…	10	31
1q42	3	2	…	4	…	…	9	28
2p21	4	…	1	4	…	…	9	28
2p13	1	…	1	3	…	…	5	16
5q31	2	…	…	3	…	…	5	16
5q33…34	1	…	…	4	…	…	5	16
9q32…34	3	…	…	4	…	…	7	22
14q32	3	…	…	5	…	…	8	25
16q22…24	1	…	…	4	…	…	5	16
19q13.1	5	…	…	8	…	…	13	41
Deleted (green/red < .5):								
16q12…13	5	…	…	3	…	…	8	25

elsewhere (Korenberg and Chen 1995). Three-color images including the CGH ratio were captured by a Photometrics cooled charge-coupled device (CCD) camera (CH250) and Oncor Imaging analysis system equipped with a Zeiss 135 Axiovert fluorescence microscope. First, the images of reversed-banded chromosomes were acquired by means of Zeiss filter set 5 (exciter 400–440 nm). To do this, the counterstain was used at low concentration for a short time, so that the banding faded rapidly after image capture by the CCD camera. The hybridization signals then were captured independently by means of a triple-bandpass filter (P/N 61002; Chroma Technology), with no interference from the banding signal. A second banding image, of lower resolution, was then collected by means of a long exposure through the same triple-bandpass filter and was used to register the high-resolution–banding image with the CGH images. The three images were then independently recalled and were evaluated both for the existence of amplification (comparison of tumor DNA with germ-line DNA) and for its precise location on the banded chromosomes. To assure proper registration (X-and-Y–shift pixel number), the second image, representing both the banding and FITC signals simultaneously, was acquired, with longer exposure times, by means of the second dichroic filter (497 nm; exciter filter FITC-HQ) that also was used to acquire the final CGH signals. This second image procedure effectively quenches the chromomycin banding signals (data not shown) and prevents the chromomycin emission from interfering with the subsequent acquisition of the CGH-signal ratios. The FITC and rhodamine images were then taken for CGH-signal ratios calculation by means of the same dichroic/emitter set but with a different excitation filter (P/N 81P490 for FITC-SB and P/N 81P570 for Texas Red-SB), to obviate the effects of movement of the dichroic filter. To define the pixel shift between dichroic sets and to generate accurate band assignments, the FITC/chromomycin signal was superimposed on the chromomycin signal, and this position used to superimpose the CGH images on the banding patterns. Finally, for illustration, the banding image was shifted to show the amplified or deleted regions clearly.

CGH-Signal Ratio Analysis

The Oncor Imaging system (version 1.6) was used for CGH-signal ratio analysis. Images were acquired and superimposed for the tumor and control DNA hybridizations on a total of 10-20 cells for each tumor DNA and cell-line DNA and were evaluated for technical adequacy (i.e., strength and smoothness of hybridization signals from both the tumor and control DNAs—as well as quality of the chromosomal spread, including very well-spread chromosomes at the 500-band stage). The images of four representative cells of each tested DNA were then selected and saved for detailed visual analyses and for digitized CGH-signal ratio determination, resulting in a total of 140 cells included in the analyses (table 1). The analysis of CGH-signal ratio and color was done by an investigator who was blind to the tumor types and to the previously amplified or deleted regions. Regions were visually considered as candidates for amplification or deletion by virtue of their intensity of signal and yellow color on the registered images of tumor DNA versus normal control DNA. For each saved cell, the putatively aneuploid chromosome bands were then con-

firmed by digitized CGH images as amplified or deleted and were then recorded for each cell. This was done in part to accommodate the inability of the available Oncor Imaging system CGH software to average signals across multiple cells. Ratios >1.5 were considered as indicative of amplification whereas those <0.5 were considered as indicative of deletion, when the observed ratio was ~1.0 for the remainder of the chromosome. However, significant ratios acquired from the telomere and centromere regions were not considered positive, in view of the existence of artificial ratios in these regions, although this may result in missing some rearrangements. Furthermore, because regions initially were selected visually, some more-subtle changes in copy number may have gone undetected.

Chromosome 2 BAC-Library Construction

Further analysis was focused on the highly amplified region of chromosome band 2p21. To assist in the mapping and defining of genomic clones for the regions, a chromosome 2 BAC map was constructed as described elsewhere (Wang et al. 1994). This collection was enlarged to 373 by the addition of 32 members. Each BAC was mapped, by FISH, to a 2–6-Mb region of chromosome 2.

Size Estimation of Amplified Region, by Dual-Color FISH

The cohybridization of the closest flanking BAC clones that were not amplified was performed according to procedures described elsewhere (Korenberg and Chen 1995). BAC 1D9 was biotin labeled, and BACs 401E9 or 2B5 were digoxigenin labeled. The probe combination of 1D9 with either 401E9 or 2B5 was hybridized simultaneously to chromosome slides made with a sample from a normal male, and images were collected as described above. In addition, we have searched the Whitehead sequence-tagged-site (STS)–map database (Whitehead Institute for Biomedical Research/MIT Center for Genome Research) to which our BAC resource was linked. The distance between two flanking STSs in the region was defined on a radiation-hybrid (RH) map and the genetic maps (Whitehead Institute for Biomedical Research/MIT Center for Genome Research).

BAC Characterization

Two BAC libraries (Shizuya et al. 1992; Wang et al. 1994) and a single P1-derived artificial chromosome (PAC) library were used (Research Genetics [Ioannou et al. 1994]). The BAC library was arrayed at a high density of 8 × 12-cm nylon-membrane filters. The majority of the BAC and PAC clones in the region were identified, by means of a single-copy fragment derived from PKCε cDNA, as hybridization probes (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data). BACs and PACs were prepared from *Not*I/*Eco*RI digests, and BAC-to-BAC or BAC-to-PAC Southern blotting was performed as described above. A total of 18 positive clones were analyzed by FISH, and only those both mapping to 2p21 and amplified on double minute chromosomes (DMs) were characterized further.

BAC Sequencing and Gene Identification

BAC 1D9 was sequenced by means of a modified shotgun sequencing strategy, as described elsewhere (Chissoe et al. 1997; Wilson and Mardis 1997). This entailed initial sequencing of random plasmid or M13 subclones. BAC DNA was sonicated, and the resulting end-repaired fragments were ligated to either M13 or plasmid vectors. M13 DNA was prepared by the ThermoMAX method, which involved nonionic detergent denaturation of polyethylene glycol–precipitated phage particles (Mardis 1994). Plasmid DNA was prepared by the AGCT 96-well boiling Miniprep method (Advanced Genetic Technologies). Sequitherm-catalyzed (Epicenter Technologies) fluorescent-dye–primer cycle-sequencing reactions (Fulton and Wilson 1994) using plasmid or M13 template DNA were electrophoresed by ABI 373A Sequencers (Perkin-Elmer, Applied Biosystems), with which the data automatically were collected and analyzed. Data processing was performed by OTTO script (L. Hillier, personal communication), which assessed trace quality, performed vector clipping, and assembled sequence data by XBAP (Dear and Staden 1991). Gaps were closed by directed dye-primer or dye-terminator sequencing reactions, by means of mapped subclone DNA or DNA fragments generated either by PCR amplification of BAC DNA or by mapped subclone DNA. The sequence was edited within the XBAP-database interface. All regions of the finished sequence were covered by more than one subclone, either sequenced in opposite orientations or by means of alternative chemistries. Restriction-fragment–digestion analysis was used to verify final sequence assembly. The sequence was then screened against GenBank 2 EMBL (BLAST X1.47mp). The known candidate genes lying within 2p21 and 2p13 also were examined for amplification in WRO cells, by Southern blotting.

Results

DNA-Copy-Number Changes Detected by CGH

The DNAs isolated from a total of 33 tumors and from two cell lines were analyzed by CGH (see the Appendix). To minimize false-positive results, the list includes only regions that were found to be aneuploid in two or more tumor DNAs and in two or more cells for

each given DNA. The ratios from the control experiments showed no changes >1.2, for any chromosomal region. All test DNAs were used in at least two independent experiments—with the exception of two follicular carcinomas (J3 and J4), which, because of limited DNA amounts, allowed only a single experiment. Because additional experiments might have confirmed the regions putatively amplified but observed only in single cells, these regions are listed here: band 2p25, in single cells of two of four DNAs; and bands 2p23, 2q35, 3q21-23, 7q11-21, 8q12-21, and 14q11.2-21, each in only one of four DNAs.

For 26 tumor DNAs and one cell-line DNA, the visual inspection suggested a number of possible amplifications or deletions, each of which then was confirmed by the digitized CGH ratios, on the basis of the criteria described in the Material and Methods section (numerical data are not shown). These chromosomal regions are summarized, by chromosome band, in table 1. The total frequency of amplification for a given region is defined as the ratio of the number of test DNAs showing this region to be amplified, versus the total number of tumor or cell-line DNAs tested. For five of the tumors (one anaplastic tumor, one follicular carcinoma, two papillary carcinomas, and one follicular adenoma), no regions suggesting an amplification were detected either by the visual inspection of the initial 10–20 cells or on the four saved images. Three additional tumors showed poor hybridization and, because of the limited amount of tumor DNA, were not retested.

Eleven regions revealed putative amplifications in 16%–41% of the tumor and cell-line DNAs, and one revealed a deletion in 25% of DNAs (table 1). The small number of tumors in each class limited the extent to which phenotype-specific differences in amplification patterns could be detected. All tumor types revealed a similar pattern of regions amplified—with the exception of the follicular carcinomas, for which the small amounts of DNA limited the data that could be collected. Chromosome band 19q13.1 revealed the highest incidence of putative amplification (41%), followed by bands 1p36 (32%), 1p34, 1q42, 2p21 (all seen in 28% of DNAs), and 14q32 (25%). Chromosome band 16q12-13 appeared consistently decreased in signal (25%).

The amplification of 2p21 and 2p13 seen in the WRO cell line also was observed in a significant proportion of thyroid tumors, providing the opportunity to further narrow the region containing a putative gene involved in thyroid tumorigenesis. However, further definition of putatively amplified regions was quite limited in those tumors that were unaccompanied by archival sections or cell lines. The 2p21 region was therefore analyzed more extensively in WRO cells.

The WRO cell line originated from a follicular carcinoma and, by standard cytogenetic analyses, was shown to harbor DMs. Two of the 32 tumors, as well as the WRO cell line, revealed amplification of band 2p13. Figure 1 illustrates a reverse-banded chromosome image side-by-side with the corresponding tumor-DNA hybridization image obtained by use of WRO DNA.

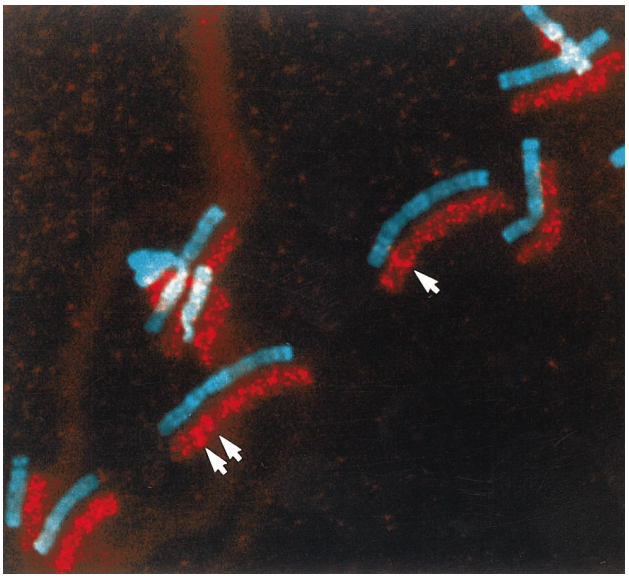

Figure 1 Mapping of amplifications on chromosome 2 in the thyroid follicular-carcinoma cell line, WRO, by CGH. Illustrated is a reverse-banded chromosome image side-by-side with the corresponding tumor-DNA hybridization image obtained with WRO DNA. The amplified region is indicated by arrows aligned with chromosome bands 2p21 and 2p13.

Molecular Definition of the Amplified Region on Chromosome Band 2p21

In order to define more precisely the regions amplified on chromosome bands 2p21 and 2p13, as well as their relationship to the DMs, the regions were further analyzed, by FISH of BACs. To test for the presence of sequences throughout chromosome 2, a series of BACs were mapped, at random, from a chromosome 2–specific BAC library by FISH (Wang et al. 1994) and were combined with chromosome 2 BACs derived from a whole-genome, randomly mapped BAC resource (Chen et al. 1996). The distribution of chromosome 2 BACs is illustrated in figure 2. A total of 87 chromosome band–specific BAC DNAs from this BAC resource then were tested independently; their locations are shown in figure 3; of these, 17 were located at regular intervals spanning the chromosome, 38 mapped to chromosome band 2p21, 3 to the border of 2p22-21, 2 on the border of 2p21-p16, and 27 to chromosome band 2p13. The results indicated that, of the 21 BACs that had been mapped to 2p21 at random, BAC 1D9 and three additional BACs generated signals on the normal chro-

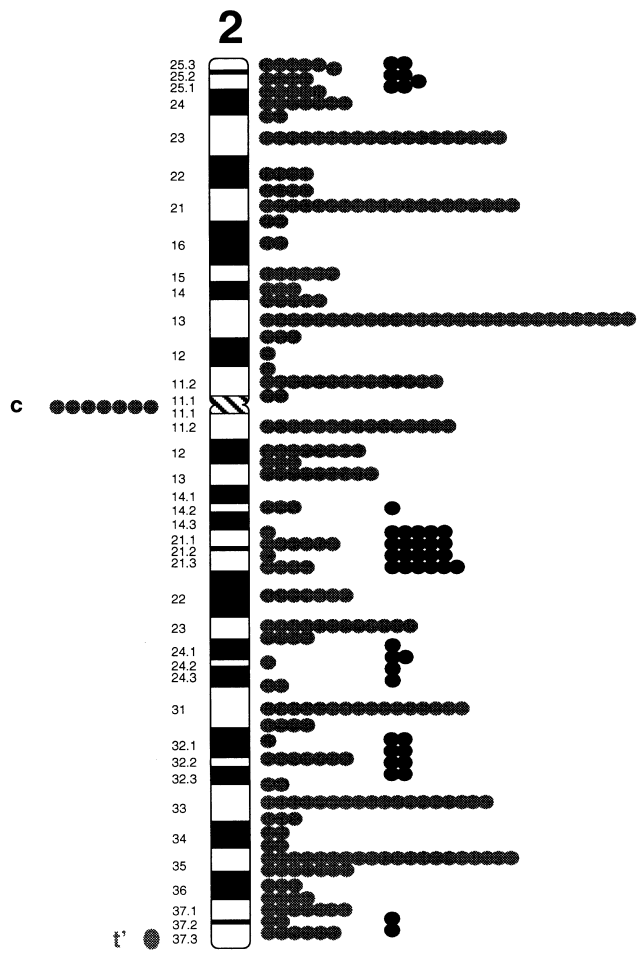

Figure 2 FISH-mapped chromosome 2 BAC resource. The ideogram illustrates a total of 373 BACs randomly mapped on chromosome 2, at 650-band resolution, by FISH. Each dot represents a single BAC. The dots on the left side represent BACs mapping in the regions of centromere (c) and telomeres (t').

mosome 2, as well as on the regions amplified on DMs in the WRO cell line (fig. 4). This yields an initial BAC density of ~1/500–1,000 kb, when it is assumed that 2p21 contains 10–20 Mb of DNA. Although chromosome preparations from early passages of WRO revealed that 30%–40% of cells contained 40–70 hybridization signals, in later passages this decreased to 15%–20% of cells containing 12–50 hybridization signals. None of the 27 BACs mapped at random to band 2p13–generated signals on the DMs.

Estimation of the Molecular Limits of the Amplified Region, by Dual-Color FISH and Genomic Map Distance

To determine the BACs most closely flanking the amplified region, nonamplified BACs mapping on band 2p21 were tested in pairwise combinations with the amplified BAC 1D9, by multicolor FISH, in metaphase and interphase. The results, shown in figure 5, revealed clearly ordered but overlapping signals for the centromeric (BAC 401E9) and telomeric (BAC 2B5) borders. The closeness but clear separation of signals from this pair of flanking BACs suggested a maximal distance of 3–6 Mb.

The second approach used to determine the maximum size of the amplified region was to estimate the distance between STSs linked to nonamplified BACs that flanked the amplified region. This was done by reference to the Whitehead STS (Whitehead Institute for Biomedical Research/MIT Center for Genome Research) and RH maps. Of the 38 BACs mapping on band 2p21 and tested for amplification, 3 were found to be linked to STSs incorporated within the Whitehead maps (Whitehead Institute for Biomedical Research/MIT Center for Genome Research). These included the amplified BAC, 1001P2, which carries the STS W5623, and two flanking nonamplified BACs, 388C11 and 401E9, which are linked to the STSs WI8407 and WI4284, respectively. The distance between these markers is 1.6–3.2 Mb, as estimated on the basis of the RH map (3.7 cR/Mb), and 8–10 Mb (1 cm/Mb), as estimated on the basis of the Genetic Map.

BAC Characterization and Amplicon

In order to provide further markers with which to confirm the molecular nature of the amplification event in 2p21, 18 of 38 BACs mapping in 2p21 were obtained from library screens by means of whole-BAC DNA from 1D9 and 3′ and 5′ PCR-generated fragments of PKCϵ, since exon 1 of this gene was the only expressed fragment found to be present within the amplified BAC 1D9 (see below). Nine of these BACs were clearly amplified on the DMs of WRO, for a total of 13 (including the 4 at random). Preliminary results with these BAC DNAs and the PKCϵ cDNA used as probes on BAC Southern blots revealed a minimal amplified region of 420 kb, established on the basis of the size of all nonoverlapping amplified BACs.

BAC Sequencing and Gene Identification

As a first approach to determining the candidate genes whose amplification was involved in thyroid tumorigenesis, BAC 1D9 was sequenced (GenBank accession number U51244) and was found to consist of 67 kb. BLASTX analysis (Altschul et al. 1990; Chissoe et al. 1997) of the completed sequence revealed database identity with human (Basta et al. 1992), rat (Ono et al. 1987), mouse (Schaap et al. 1989), and rabbit (Ohno et al. 1988) PKCϵ. The amino acid translation of 1D9 nucleotides 13230–13577 matched amino acids 1–116 of each mentioned PKCϵ entry, with 100% identity. Presumably, this represents the first exon of the human PKCϵ gene. Additional database similarities identified

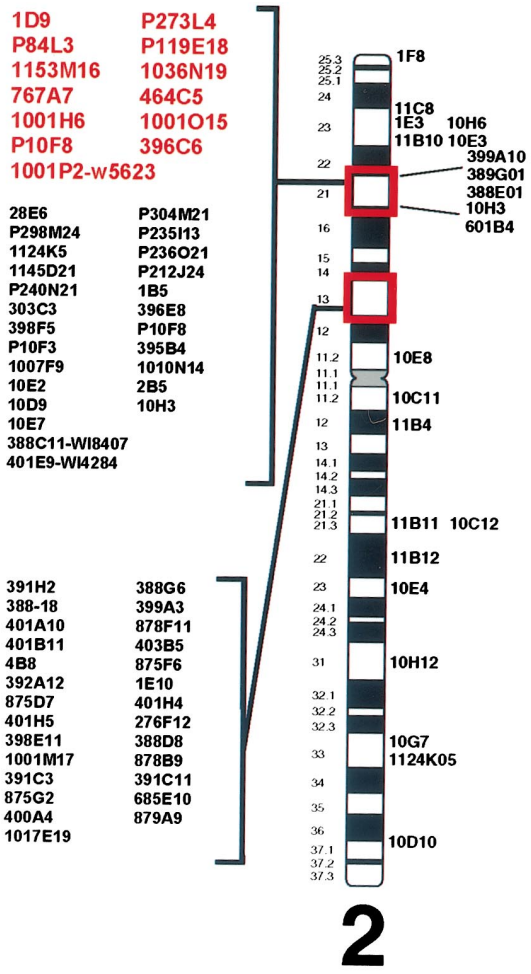

Figure 3 BACs amplified in the thyroid follicular-carcinoma cell line, WRO. The ideogram shows the cytogenetic locations of a total of 87 chromosome-band–specific BACs tested on WRO. The amplified BACs are indicated (*red*).

were to other members of the protein kinase family from human, mouse, and *Caenorhabditis elegans*. The GC content within a 2-kb region immediately upstream of the exon was 68.2%, representing a putative CpG island, compared with an overall GC content of 44.5% for the entire clone. This exon was then mapped back to 1D9, and its amplification was confirmed by Southern blot analysis in the WRO cancer-cell line (fig. 6). Further exploration of both the frequency of PKCϵ structural abnormalities in thyroid cancers and its possible functional consequences will be reported in a separate article (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data).

As a second approach to determining the genes in the amplified regions, Southern blot analyses with probes for genes known to map to the region of 2p13 (the MAD oncogene, TGFα, and the RAL avian reticuloendotheliosis viral oncogene homologue) and 2p21 (the carbamoyl phosphate synthetase asparate transcarbamylase and dihydrocrotase gene [CAD], the ras guanine nucleotide-exchange factor, SOS [Son of Sevenless], and the DNA-repair enzyme hMSH2) indicated that they were not involved in the amplification event (data not shown). Although exon 1 on PKCϵ was found within BAC 1D9, it was not detected by exon trapping, presumably because of the lack of splice-donor/-acceptor sites in the 5′ end of the first exon of this gene (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data). Since BAC 1D9 contains 12 kb of the PKCϵ upstream region, it may also contain the ends of the amplicon for this amplification event. To determine this, the two ends of BAC 1D9 were sequenced and were tested for amplification on Southern blots of WRO genomic DNA, and both were found to be amplified.

Identification of a Chimeric PKCϵ in the WRO Cell Line

To investigate the possible involvement and/or rearrangement of PKCϵ in the amplification events, a full-length human PKCϵ cDNA was hybridized to Southern blots containing DNAs from the WRO cell line, the anaplastic thyroid carcinoma cell line, and normal tissue (fig. 6). These demonstrated that the PKCϵ gene had undergone rearrangement and amplification in the WRO cells, since there were additional bands found in the

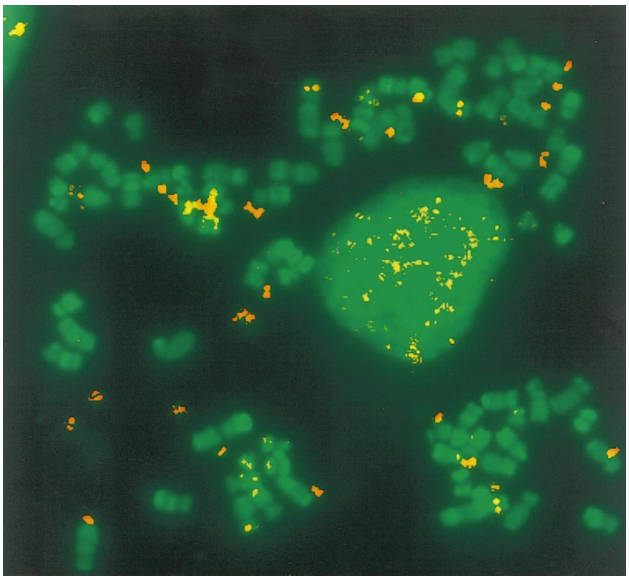

Figure 4 Hybridization of BAC 1D9 on WRO chromosome preparations, showing a distinct pattern of amplification. In addition to the FITC signals (*yellow dots*) detected on the three copies of chromosomes 2, on band 2p21, clusters of signals were detected both on DMs and in adjacent interphase nuclei.

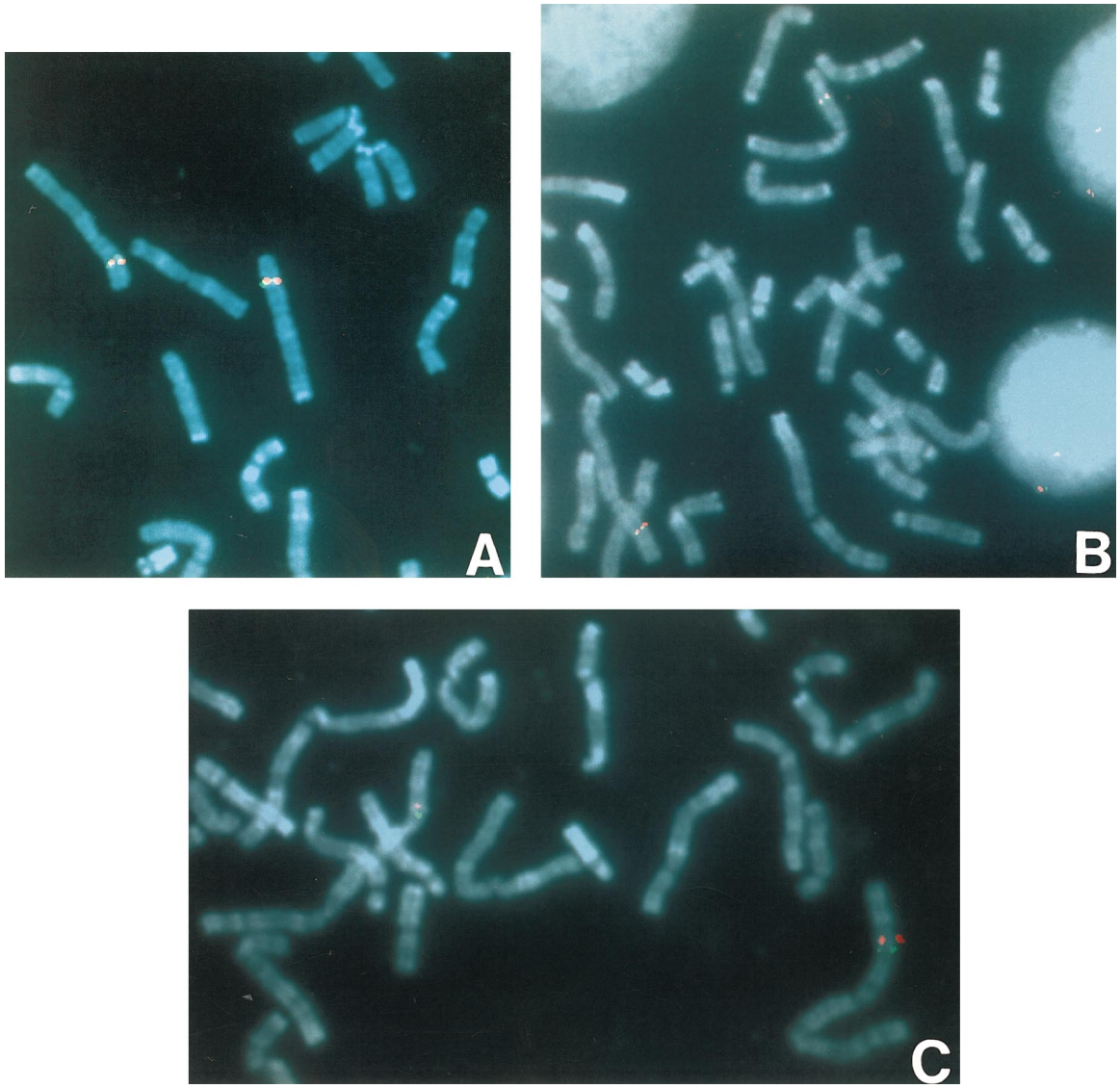

Figure 5 Dual-color FISH mapping of BACs most closely flanking 1D9 on 2p21. *A,* BAC 2B5 (*pink*), mapping distal to but partially overlapping BAC 1D9 (*green*). *B,* BAC 401E9 (*green*), mapping proximal to but overlapping BAC 1D9 (*pink*). *C,* Two flanking BACs (BAC 401E9 [*green*] and BAC 2B5 [*pink*]), which map close to one another but are clearly separated.

WRO cell line that were not found in normal tissue (fig. 6). The detailed characterization of this abnormality and of its phenotypic impact is shown in the work of J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, (unpublished data). These results provided the basis for further investigation of PKCε as a candidate gene for thyroid tumorigenesis.

Discussion

There have been major gaps in our understanding of the biological behavior of thyroid tumors, resulting in diagnostic inaccuracy and uncertainty as to the best treatment for the various forms of the disease. There have been no previous reports of homogeneously staining regions or DMs representing highly amplified chromosomal regions (Schimke 1988) in any thyroid neoplasms, as determined by cytogenetic analysis (Fagin et al. 1993).

The power of the approach presented in this study is in the ability to begin with a single tumor-derived cell line containing amplified regions and a series of tumor DNAs and to use CGH coupled with a high-density mapped array of BACs and genomic sequencing to define both the regions of amplification and the genomic re-

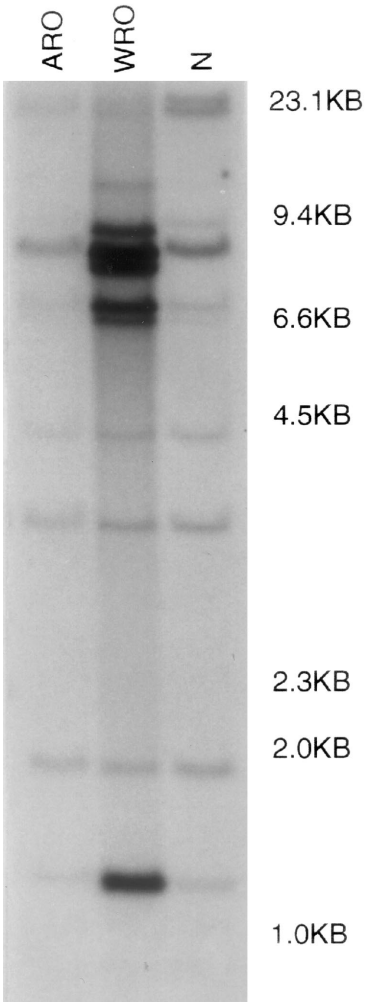

Figure 6 Confirmation of amplification event on WRO, by Southern blot hybridization of DNA from normal lymphocytes (N) and thyroid carcinoma cell lines (ARO and WRO), digested with *Hin*dIII and probed with full-length PKCε cDNA. Note the amplification of selected bands and of aberrantly sized restriction fragments, indicative of a rearrangement. As expected, no amplification is seen in ARO.

agents with which to identify and test the genes involved in the pathways of tumor formation. Our approach has rapidly defined a BAC, 1D9, and has mapped the gene for PKCε, which subsequently has been shown to have significant phenotypic effects consistent with a tumor-promoting action in thyroid cells (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data). The mapped BAC resource for chromosome 2 used for the initial analysis was composed of ~1 BAC/Mb (245 BACs/285 kb), resulting in the ability to detect ~70% of duplications >1 Mb, under the assumption of random distribution. Use of a whole-genome array of BACs at this density would result in the ability to detect most amplification events throughout the genome and to flank closely most chromosomal rearrangements associated with tumorigenesis. Such a resource has been generated, is visible at the Website of the corresponding author of this article (J.R.K. [Molecular Genetics Labs at CSMC]), and, in part, has been integrated with the STS and physical maps of the human genome. This resource will be of great value for the definition of chromosomal rearrangements associated with neoplasia and will be useful both for gene discovery and, subsequently, to guide diagnosis, prognosis, and treatment.

High-Resolution Banding for CGH

This article reports the development and application of a banding technique employing chromomycin and distamycin for use with CGH. In contrast to gene mapping, in which it is desirable to view the banding pattern simultaneously with the hybridization probe, the images generated for CGH, by both the tumor DNA and the control DNA, must be captured in the absence of light from any other source but, nonetheless, must be registered with the banding pattern. Previously, this requirement was satisfied by use of three different dyes. For example, most current techniques rely on DAPI for banding (Kallioniemi et al. 1994*b*), which provides a low-resolution banding pattern. We therefore modified our higher-resolution, chromomycin A3/distamycin reverse-banding technique, so that the banding image was captured accurately but independent of the hybridization signals. This technique permits high-resolution assignment of amplified and deleted regions to single human chromosome bands by CGH alone and provides a means for defining the more appropriate BACs to be tested for amplification, as described in the Material and Methods section.

Amplification and Deletions by CGH

We have reported the analysis of 33 thyroid-tumor DNAs and two cell-line DNAs and have detected 11 commonly amplified regions plus a single deleted region that were defined by CGH. It is possible, although it has not been determined by this study, that each of these regions contains genes that are amplified in thyroid tumors. These data provide the basis with which to focus future studies of candidate-gene amplification in thyroid tumors. Although we have focused on the 2p21 locus, it is of interest that amplification 2p13-15 has been reported in an extranodal diffuse large-cell lymphoma (Houldsworth et al. 1996). However, in the present study, all of the known oncogenes in this region were excluded on the basis of the lack of both amplification and overexpression in the WRO cell line, as described in the Results section.

Previous reports have used CGH to document DNA

amplifications of similar chromosome bands (e.g., 1p36) or chromosome arms, including 1q, 2p, 5q, 9q, 14q, 16q, and 19q, from a variety of tumors, including lymphomas, colorectal tumors, neuroectodermal tumors, liposarcomas, oral squamous-cell carcinoma, and prostate cancers (Matsumura 1995; Bentz et al. 1996; Bockmuhl et al. 1996; Ried et al. 1996; Schutz et al. 1996; Szymanska et al. 1996). However, these reports led to the identification of candidate genes that previously had been known to exist in the region. In contrast, use of a dense BAC clone array, has, as shown in this study, provided the critical link to allow the definition of previously unmapped candidate genes that are to be identified by a combination of CGH and positional cloning. It may be of interest to determine the relationship between the 2p21 amplification event involving PKCε, reported here, and the chromosome 2 inversions associated with a previously reported papillary carcinoma (Lehmann et al. 1997). It will be possible to use the current approach, employing the BAC resource to define minimal regions likely to contain oncogenes or tumor-suppressor genes, for study of the progression of these tumors. In the tumor collection used in the present study, more-detailed analysis of aneuploidy was limited by the lack of tumor materials with which to test candidate BACs.

Our study may be less quantitative and less sensitive for analysis of deletion, because the CGH software (Oncor Imaging) available for this project was not capable of combining the data across cells. On the other hand, losses of chromosome 16q also have been seen in high-grade ovarian tumors (Iwabuchi et al. 1995) and in prostate cancer (Joos et al. 1995; Cher et al. 1996), indicating the possible presence of tumor suppressors in the region.

Molecular Definition and Size Estimation of the Amplified Region

Our data revealed that a region on 2p21 was highly amplified in the WRO cell line with DMs, with a lesser amplification event on 2p13, providing the opportunity for a further molecular analysis of these regions. The present study, therefore, focused on the analysis of these regions. Furthermore, the DNAs from eight other tumors also revealed amplification of the same chromosomal band, 2p21 (table 1), suggesting that this region may harbor a gene whose overexpression might be involved in tumor progression. To further analyze the amplification event, a 373-member chromosome 2 BAC resource was generated, and a subset was used. These BACs were first mapped to their respective loci on chromosome 2 by multicolor FISH, as illustrated in the reports by Wang et al. (1994) and Chen et al. (1996). However, only a subset of the BACs mapped to 2p13 and 2p21 were used to investigate amplification in the WRO cell line. The variability of the signals seen on different DMs within a single interphase in the WRO cell population suggests the existence of a minor cell population with both a higher copy number and a smaller amplicon size. The results firmly established amplification of this region on the DMs. Furthermore, there was no evidence of the involvement of any other region of chromosome 2 in the amplification event, in contrast to the results with chromosome 20 that are seen in the study of breast cancer (Kallioniemi et al. 1994*a*).

Both of the map-based distance estimations (i.e., RH and genetic-marker map) represented averages for the entire human genome and were associated with significant variation for a given region. It is of interest that the estimate derived from the molecular cytogenetic data fell between these two and, given the lesser variation in chromosome condensation, likely represented a reasonable measure. However, it is important to note that all estimates may be gross overestimates of the true size of the amplified region in the DMs seen in WRO, because, in each case, the flanking BACs or markers were located at an unspecified distance from the amplified region. It is of note that these estimations of maximal size of the amplified region are small when compared with those determined by most previous CGH analyses, which included a number of bands or an entire chromosome arm. It is important to note that the resolution obtained in this study is possible for the entire genome using the currently available resource. Given the density of this resource, it represents an alternative to chromosome microdissection, for definition of the regions amplified in DMs (Guan et al. 1994).

BAC Characterization and Gene Identification

The amplified contig defined in this study may contain the ends of the amplicon and, therefore, could provide the tools with which to isolate further candidate genes. This could be established by extension of the contig and evaluation of cytological preparations that may be more sensitive for detection of low-frequency amplification.

Further evaluation of the structure and expression of the PKCε region in WRO revealed a structural rearrangement involving the gene (fig. 6), which resulted in the overexpression of a hybrid molecule, as seen on northern analyses (data not shown; J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data). The altered function of this molecule in the signal-transduction cascade may have been a significant event in the transformation of this tumor clone (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, and J. A. Fagin, unpublished data). This possible role for PKCε in thyroid tumorigenesis is supported by pre-

vious reports, which implicated abnormal PKCϵ expression in endocrine tumors (Kuranami et al. 1995).

It is of interest that significant amplification of 2p21 was clearly observed by CGH only in the cell line carrying DMs. Taken together with the observation of a genomic rearrangement and expression of a truncated gene product, this may indicate that mutation or rearrangement—rather than overexpression—of PKCϵ may be responsible for its role in thyroid tumorigenesis. This finding indicates that identification of uncommon events such as amplification may point to genes whose role in tumorigenesis may involve more-complex mechanisms.

In summary, CGH has been successful in identification of candidate genes likely to be involved in tumorigenesis. At present, only a few human chromosomes (2, 6p, 11p, 16, 19, 21, X, and Y) are relatively well covered with large-fragment vector arrays—such as cosmids, BACs, or PACs—that are suitable either for use in definition of amplified regions or to be fixed, as CGH targets, as arrays on glass chips. Our current chromosome 2 resource (Wang et al. 1994), as well as our integrated genomewide BAC and PAC resource (Chen et al. 1996), represents a very powerful tool for this purpose. Four aspects of the current approach are of importance. First, the higher-resolution R-banding method facilitates the assignment of the amplified region to a chromosome band and links the amplified region with the physical map. Second, the availability of chromosome-band–specific BACs and PACs that represent >25% of the genome greatly facilitates the speed with which a subset of BACs mapping in an amplified region can be determined. Third, the existence of the integrated BAC and PAC resource linked to STS information establishes the link to both the physical and genetic maps, which, in turn, provides markers for rapid completion of a BAC and PAC array and for establishment of the gene/EST candidates for roles in tumorigenesis. Fourth, the current large-scale sequencing used in combination with this approach provides the ability to rapidly identify candidate genes involved in tumor progression.

It is of importance to note that these technical advances are equally powerful in translating the rapidly developing application of CGH to genomic DNAs immobilized either on glass slides or, ultimately, on computer chips (Cheung and Nelson 1996). The establishment of mapped, integrated, and partially ordered large-fragment (BAC or PAC) clone arrays that represent a large proportion of the human genome becomes essential both for maximization of the probability of finding amplification events and for rapid translation of these into a series of candidate genes. In the present study, the next steps have been taken and the entire approach has been illustrated: the sequencing of an entire BAC located in the amplified region has revealed the gene encoding PKCϵ, which has led to the demonstration of both a genomic rearrangement and overexpression of the chimeric gene product. As described elsewhere (J. A. Knauf, R. Elisei, D. Mochly-Rosen, T. Liron, X.-N. Chen, J. R. Korenberg, J. A. Fagin, unpublished data), the product may selectively impair programmed cell death in thyroid cells, thus demonstrating that the genetic-amplification event detected by CGH coupled with the mapped BAC resource has identified a legitimate candidate tumor-promoting gene that likely plays a role in oncogenesis.

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Appendix

List of Thyroid Tumors Tested

Follicular Adenoma (n = 10)		Follicular Carcinoma (n = 5)	Papillary Carcinoma (n = 18)				Anaplastic Carcinoma (n = 2)
60	R15	WRO	146	R2	R7	R12	ARO
A9	R16	FRO	162	R3	R8	A4	82I
A19	R17	J3	163	R4	R9	NPA	
R13	R18	J4	165	R5	R10		
R14	115	R19	R1	R6	R11		

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Molecular Genetics Labs at CSMC, http://www.csmc.edu/genetics/korenberg/korenberg.html

Whitehead Institute for Biomedical Research/MIT Center for Genome Research, http://www-genome.wi.mit.edu/

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