Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1616–1620, March 1992
Medical Sciences

Characterization of an inversion on the long arm of chromosome 10 juxtaposing *D10S170* and *RET* and creating the oncogenic sequence *RET*/PTC

(locus identified by probe H4)

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Communicated by J. Edward Rall, October 4, 1991

ABSTRACT **_RET_/PTC is a transforming sequence created by the fusion of the tyrosine kinase domain of the _RET_ protooncogene with the 5′ end of the locus _D10S170_ designated by probe H4 and is frequently found activated in human papillary thyroid carcinomas. _RET_ and _D10S170_ have been mapped to contiguous regions of the long arm of chromosome 10: q11.2 and q21, respectively. To identify the mechanism leading to the generation of the oncogenic sequence _RET_/PTC, a combined cytogenetic and molecular analysis of several cases of papillary thyroid carcinomas was done. In four cases the results indicated that these tumors had _RET_/PTC activation and a paracentric inversion of the long arm of chromosome 10, inv(10)(q11.2q21), with breakpoints coincident with the regions where _RET_ and _D10S170_ are located. Therefore, a chromosome 10q inversion provides the structural basis for the _D10S170–RET_ fusion that forms the hybrid transforming sequence _RET_/PTC.**

In some forms of cancer, chromosome rearrangements have provided the structural basis of their molecular pathogenetic mechanisms. In particular, in hematologic malignancies, chromosomal translocations result in deregulation of the expression of the cell-growth-related genes c-*MYC* and *BCL2* in Burkitt and in follicular lymphomas, respectively (1–3). In other instances, the translocation may generate chimeric transforming transcripts as in the case of the *BCR–ABL* fusion protein found in chronic myelocytic leukemia (4).

In solid tumors, however, only rarely have chromosome aberrations been analyzed at the molecular level. The most relevant examples include the chromosomal deletions found to inactivate one or both copies of tumor-suppressor genes—such as *RB* in retinoblastoma, *WT1* in Wilms tumor, and *DCC* in colon carcinoma (5).

The only example of generation of chimeric transforming sequences in solid tumors is the activation of c-*MET* on chromosome 7 by the *TPR* gene that maps to chromosome 1 (6). However, this event, most likely a chromosomal translocation, has been generated *in vitro* by treatment of the original human osteogenic sarcoma cell line (HOS) with a chemical carcinogen (7). In other cases the rearrangements giving rise to the transforming fusion products have been generated during the transfection procedures, as in the case of *RET* (8, 9). When a similar genetic event occurred somatically in the original tumor DNA, like the fusion between the nonmuscle tropomyosin sequence and the tyrosine kinase domain of proto-*TRK* gene (10), no chromosomal aberrations were identified to provide a model for the relative mechanism.

We have reported (11) the occurrence in papillary thyroid carcinomas of specific rearrangements of the tyrosine kinase domain of the proto-*RET* gene with an unknown "gene" *D10S170*, identified by probe H4 and originally designated the chimeric transforming sequence as *PTC*‖, but here designate it as *RET*/PTC. In two distinct sets of experiments, *RET*/PTC activation was found in ≈25% of papillary thyroid carcinomas (12, 13) and represented a tumor-specific somatic event, being detected in the original DNA of both primary tumors and metastases but not in normal cells from the same patients (12, 13). We have mapped *D10S170* to chromosome 10q21 (14, 15) and confirmed the *RET* locus at 10q11.2 (15, 16).

From one of our laboratories (R.B.J., M.A.H., and I.D.H.) the occurrence of a simple cytogenetic aberration in papillary thyroid carcinomas has also previously been reported: an inv(10)(q11.2q21) (17) with breakpoints where *RET* and *D10S170* are located. Among 18 cases of this tumor histotype, we have, to date, identified five cases that have this identical abnormality. Here we report the cytogenetic and molecular characterization of four of these tumors and demonstrate that the cytogenetic inversion has provided the structural basis for the *D10S170–RET* fusion leading to the generation of the chimeric transforming sequence *RET*/PTC.

MATERIALS AND METHODS

Chromosome Preparation. Specimens from primary tumor or metastatic lymph nodes were obtained at surgery and were histologically diagnosed as papillary thyroid carcinomas. Cases 1, 3, and 4 corresponded to pure papillary thyroid carcinomas; case 2 might be considered a diffuse sclerosing variant of papillary carcinoma. The samples were mechanically disaggregated and incubated in 0.8% collagenase type II (Sigma) at 37°C in 5% CO_2 overnight. The cell suspension was seeded and cultured in RPMI 1640 medium (Whittaker Bioproducts) supplemented with 5% fetal calf serum (Flow Laboratories), penicillin (200 units per ml), streptomycin (200 μg/ml), transferrin (10 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (5 ng/ml) (Sigma), and insulin (5 μg/ml) (Sigma).

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‖*PTC*, the symbol used originally to designate this papillary thyroid carcinoma transforming sequence, has another meaning (phenylthiocarbamide tasting) for the Human Gene Mapping Nomenclature Committee; the papillary thyroid carcinoma transforming sequence is designated *RET*/PTC.

Chromosome preparations were performed on primary culture of 10 days, according to standard techniques. G-banding was obtained by using Wright's stain.

Southern and Northern (RNA) Blot. High-M_r DNA was digested with the appropriate restriction enzymes, electrophoresed, blotted on nitrocellulose or nylon filters, and hybridized to nick-translated molecular probes, as described (11).

Northern blot analysis used 10 μg of total mRNA prepared from the cell lines indicated in Fig. 3 and was done following described procedures (11) by using as a probe a cDNA fragment corresponding to the tyrosine kinase domain of the *RET* oncogene (probe C3 of ref. 11).

Transfection Assay. The transfection assay was done on NIH 3T3 cells with high-M_r DNA obtained from the metastatic lymph node of patient 1, by following described techniques (13).

PCR. RNA amplification was obtained by following the method described by E. S. Kawasaki (18) starting from 1.5 μg of total mRNA. The map in Fig. 4 indicates the nucleotide position of the primers related to *D10S170* (196–213) and RET (485–502) sequences, respectively. The breakpoint of the *RET*/PTC sequence between nucleotides 351 and 352 and the *Eco*RI site (371) are also indicated.

RESULTS

The cytogenetic analyses of the tumor cells revealed as the specific acquired abnormality a paracentric inversion of the long arm of chromosome 10 with breakpoints at q11.2 and q21 in 15 out of 15 metaphases of case 1, 8 out of 10 metaphases of case 2, 3 out of 30 metaphases of case 3, and 29 out of 30 metaphases of case 4. Fig. 1*A* shows a representative example of the inversion from patients 1 and 2. An identical inversion was seen in patients 3 and 4. The karyotype of patient 4 has been reported in detail elsewhere (17). The peripheral lymphocytes of patients 1, 2, and 4 showed a normal 46,XX karyotype. The peripheral lymphocytes of patient 3 were not studied.

The rearrangement of the *RET* gene, leading to the generation of *RET*/PTC has been shown to occur in a 6.3-kilobase (kb) *Eco*RI fragment of the proto-*RET* gene located immediately upstream of its tyrosine kinase-encoding domain, whereas *D10S170* truncation was found at different points in different tumors (11). The DNA from the four tumors carrying the 10q inversion was analyzed by Southern blot with a 1-kilobase pair (kbp) *Bgl* II–*Bam*HI probe (Fig. 2, probe 2), specific for the 6.3-kbp *Eco*RI fragment of proto-*RET*, where all the characterized RET rearrangements have been demonstrated. The probe detected an extra band in the four tumors and not in the related normal DNA (Figs. 2 and 3*A*), indicating the tumor-specific *RET* activation (11). To demonstrate the involvement of *D10S170* sequences in the *RET* rearrangement of the four tumors, we analyzed their DNAs with the available *D10S170*-specific *Alu*-free genomic probes. In patients 2 and 3 using the 0.6-kbp *Bam*HI–*Ava* I *D10S170* probe (Fig. 2, probe 3) a rearranged band was clearly detected (Fig. 2). In patient 3 the 6-kbp *Bam*HI-rearranged *D10S170* band was of the same size as one of the *Bam*HI-rearranged RET bands. This finding indicates that the two genes are fused together. In cases 1 and 4 we were unable to show a *D10S170* rearrangement (Fig. 3*B* and data not shown). However, because case 1 provided enough tumor tissue, a transfection assay on NIH 3T3 cells was done. After 14 days 1.05 foci per μg of DNA was observed. The NIH 3T3 transformants possessed both human *RET* (Fig. 3*A*, lane NIH-T1) and *D10S170* (Fig. 3*B*, lane NIH-T1) sequences. As expected, the human *D10S170* and *RET* sequences detected in the transformants were of the same size as the *D10S170*-related fragment and the *RET*-rearranged band present in the original tumor DNA (Fig. 3*A*, lane T). As anticipated, the size of the *D10S170* fragments was not distinguishable from that of the *D10S170* fragment seen in normal DNA (Fig. 3*B*). Moreover, Fig. 3*D* shows a Northern blot obtained with a RET-specific cDNA probe; in the transformants obtained with DNA from this tumor, the *RET* oncogene was transcribed in truncated mRNAs having sizes identical to those previously detected in a *RET*/PTC-positive papillary thyroid carcinoma (11). Transcripts of the same size were detected in the same cell lines with a *D10S170*-specific probe (data not shown). A PCR analysis was performed with primers derived from both the *RET* and the *D10S170* portion of *RET*/PTC to amplify a cDNA fragment that contained the *RET–D10S170* breakpoint (11) (Fig. 4). This experiment showed that the *RET–D10S170* rearrangement, although occurring in different intronic regions, produced identical mRNA species. In fact, the *RET*/PTC cDNAs derived from NIH 3T3 transfectants of the case reported (11) and from the tumor of case 1 contained the same predicted fragment of 306 base pairs. Complementary DNA directly prepared from the tumor of patient 1 contained the same PCR fragment (Fig. 4), thus confirming *RET*/PTC activation. The specificity of the amplified bands was ascertained by *Eco*RI digestion of the PCR products that resulted in the predicted fragments (Fig. 4, bands *A* and *B*). The same result was also obtained in patient 3, confirming the above conclusions (data not shown); moreover, the specificity of the PCR was ascertained by hybridizing its products with *PTC*-specific probes and by sequencing the PCR products (data not shown).

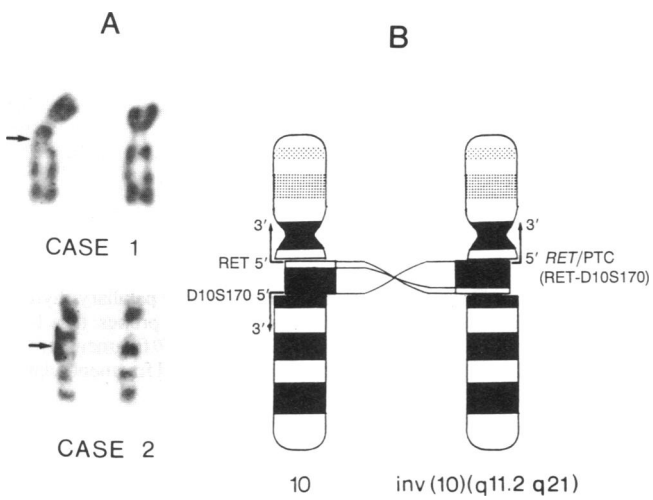

FIG. 1. Chromosome 10q inversion in papillary thyroid carcinoma. (*A*) Two representative chromosome 10 homologs from tumor cells of patients 1 and 2 showing inv(10)(q11.2q21) (arrows). (*B*) Schematic view of the paracentric inversion of chromosome 10q generating the transforming sequence *RET*/PTC. The gene order on chromosome 10q has been previously determined (16). The indicated direction of gene transcription is one of the two possibilities (divergent vs. convergent).

The cytogenetic and molecular genetic data indicate that an inversion on chromosome 10q has activated *RET*/PTC by fusing together *D10S170* and *RET* sequences. Such a mechanism would predict that in tumor DNA the sequence of the *RET* gene located upstream from the breakpoint is rearranged and fused to the 3′ end sequence of *D10S170*. The tumor DNAs from the four patients were analyzed with a probe located upstream from the breakpoint in the proto-*RET* gene (Fig. 2, probe 1), and an extra band was detected demonstrating that the 5′ end of proto-RET was rearranged in all these cases (Figs. 2 and 3*C*).

Moreover, in patients 2 and 3, we detected a rearranged band of the same size, 10.5 kbp and 7.2 kbp, respectively, with both

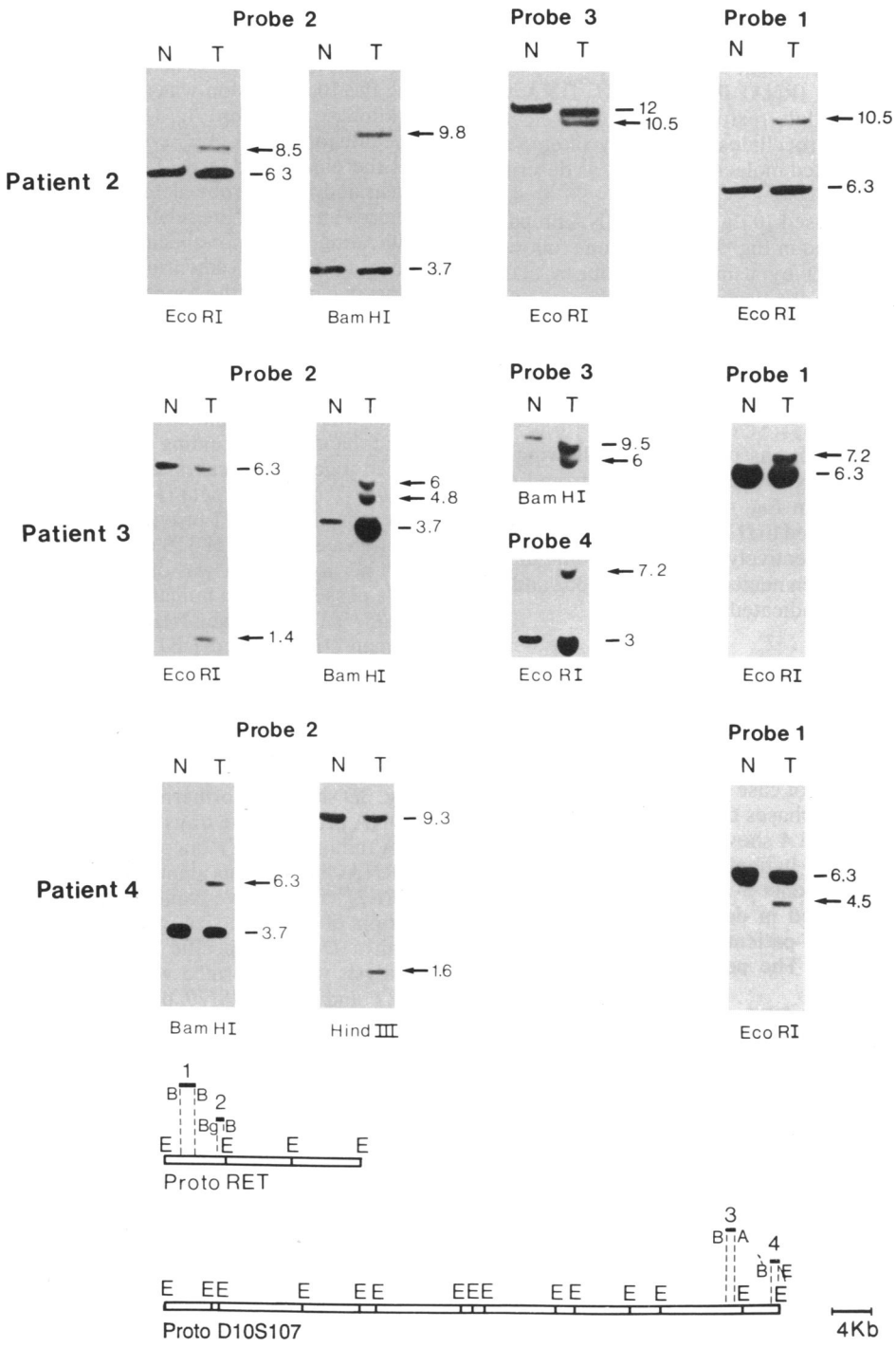

FIG. 2. Southern blot analysis of normal (peripheral blood cells) (lanes N) and tumor (lanes T) DNA of patients 2, 3, and 4 with papillary thyroid carcinomas displaying inv(10)(q11.2q21). The analysis was done after digestion with the indicated restriction enzymes and by using as probes: (*i*) a 1-kb *Bgl* II (Bg)–*Bam*HI (B) probe specific for the 6.3-kbp *Eco*RI (E) fragment of proto-*RET* (probe 2); (*ii*) a 0.6-kb *Bam*HI–*Ava* I (A) *D10S170* fragment (probe 3) for patients 2 and 3, for patient 3 also a 0.6-kb *Bam*HI–*Eco*RI *D10S170*-specific sequence (probe 4); and (*iii*) a 1.8-kbp *Bam*HI–*Bam*HI fragment related to the 5′ end of proto-*RET* (probe 1). Size of normal and of rearranged (arrows) bands is given as kbp.

3′-end *D10S170* and 5′-end proto-*RET*-related probes (Fig. 2). This band represents the fusion event reciprocal to that generating the *RET*/PTC oncogenic sequence (see Fig. 1*B*).

DISCUSSION

Oncogene activation by chromosome rearrangements has been described in some hematopoietic malignancies (1, 2, 4).

The cytogenetic and molecular analyses of the four cases of papillary thyroid carcinomas reported here provide an example of oncogene activation in a solid tumor due to an acquired chromosomal abnormality. This abnormality leads to a chimeric transforming transcript, *RET*/PTC, in analogy to the production of the chimeric protein that follows the 9;22 translocation in chronic granulocytic leukemia (4). In fact, *RET*/PTC is originated by the fusion of two unrelated sequences, a portion of proto-*RET* including its tyrosine kinase domain and *D10S170*, which provides the 5′ end promoter region (11).

The transforming sequence *RET*/PTC has been identified in ≈25% of patients with papillary thyroid carcinomas (12,

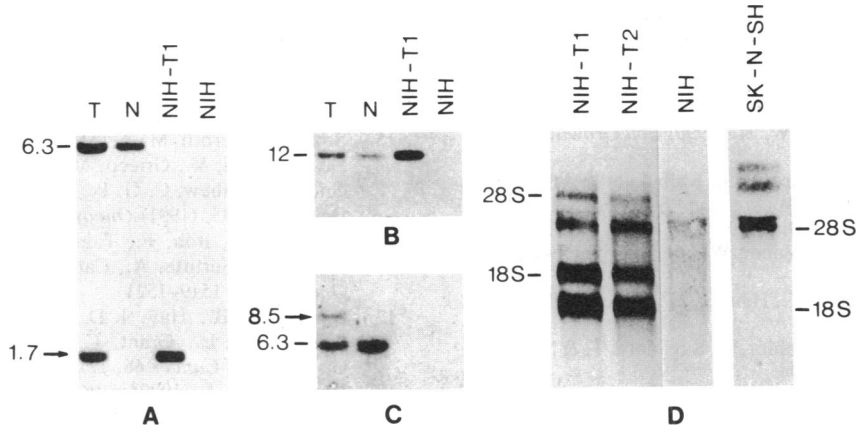

FIG. 3. Southern blot analysis of normal (lanes N) and tumor (lanes T) DNA of patient 1 and of NIH 3T3-untransfected (lanes NIH) or transformed (lanes NIH-T1) by the tumor DNA of patient 1. After *Eco*RI digestion, the DNAs were hybridized to the molecular probes described in Fig. 2 for patient 2: *RET* probe (*A*), *D10S170* probe (*B*), and 5′ end of proto-*RET* probe (*C*). The latter probe does not recognize specific sequence either in control or in *RET*/PTC-transfected NIH 3T3 (NIH-T1) because this region of proto-*RET* was truncated in the *RET*/PTC-transforming sequence formation. (*D*) Northern blot analysis of transcripts from a neuroblastoma cell line (lane SK-N-SH), representative of proto-RET mRNAs (10), NIH 3T3 transformed by the DNA from patient 1 metastatic tissue (lane NIH-T1) and from NIH-3T3 transformed by cloned and sequenced *RET*/PTC (lane NIH-T2) (11).

13). Recently, Ishizaka *et al.* (19) have cloned the same hybrid sequence, generated by *D10S170* and *RET* fusion, from a papillary thyroid carcinoma cell line. These findings make *RET*/PTC the most frequently detected oncogene in human solid tumors, immediately following the detection of oncogenes belonging to the *RAS* gene family.

A pathogenetic role of *RET*/PTC in papillary thyroid carcinomas is supported by the observation of its activation in both primary and metastatic tumor lesions and not in the normal DNA of the same patient (11–13). In addition, the normal thyroid epithelium does not express *RET*-related transcripts (11), and an inappropriate *RET* expression driven by a mouse mammary tumor virus promoter/enhancer has been recently reported (20) to form mammary and salivary gland adenocarcinomas in transgenic mice.

The present data, reporting a paracentric inversion of the long arm of chromosome 10, allow us to propose the model depicted in Fig. 1*B* for the generation of the *RET*/PTC-transforming sequence. The molecular analysis also supports the model by identifying the rearrangement bands due to the fusion event reciprocal to that generating *RET*/PTC. Because we have determined the gene order on 10q as cen-*RET*-*D10S170*-qter (15) and because the inversion resulted in the generation of functional chimeric transcripts, we can deduce that *RET* and *D10S170* are normally transcribed on opposite DNA strands. In the model we have considered only the possibility of *RET*–*D10S170* divergent transcription. However, our data cannot exclude the opposite possibility of convergent transcripts. The most relevant consequence of this model is that every case of *RET*/PTC formation in tumor cells should involve a chromosomal inversion. It has been recently reported (21) that the genes of the immunoglobulin κ light chains are assembled by somatic recombination that in some instances can include a megabase-sized chromosomal inversion, demonstrating that in physiological conditions such an event can lead to a functional product.

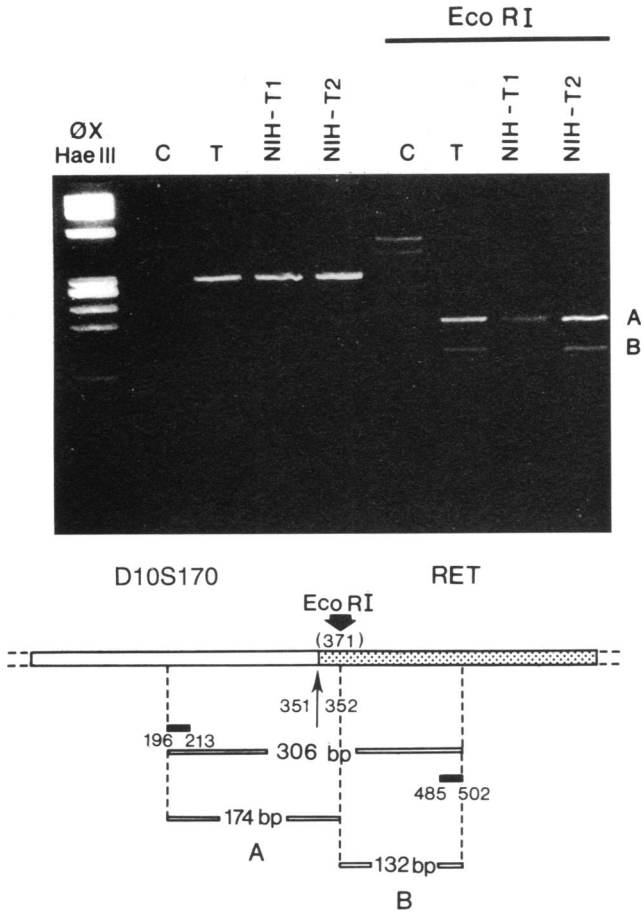

FIG. 4. PCR amplification of the fusion cDNA fragment containing the *D10S170–RET* breakpoint region, as deduced from the partial map (*Lower*) derived from *RET*/PTC sequence (11). (*Upper*) The starting RNAs were from a control human melanoma cell line (lanes C), a lymph-node metastatic specimen from patient 1 (lanes T), NIH 3T3 transformed by the tumor DNA of patient 1 (lanes NIH-T1), and NIH 3T3 transformed by the sequenced *RET*/PTC (lanes NIH-T2). Bacteriophage ØX-174 RF DNA-HaeIII digests were used as molecular size markers.

We are indebted to Dr. Silvana Pilotti for advice and for supplying the pathologic specimens. The assistance of Mr. Mario Azzini, Mrs. Carla Calderone, Mrs. Anna Grassi, Ms. Piera Mondellini, and Mr. Chris R. Shad is also acknowledged. This work was supported by the Associazione Italiana per la Ricerca sul Cancro and by the Progetto Finalizzato Biotecnologie e Biostrumentazione, Consiglio Nazionale delle Ricerche.

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