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Research Article

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Ret Oncogene Activation in Human Thyroid Neoplasms Is Restricted to the Papillary Cancer Subtype

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Abstract

We have recently reported the activation of a new oncogene in human papillary thyroid carcinomas. This oncogene, papillary thyroid carcinoma (PTC), is a novel rearranged version of the *ret* tyrosine-kinase protooncogene. Thyroid neoplasms include a broad spectrum of malignant tumors, ranging from well-differentiated tumors to undifferentiated anaplastic carcinomas. To determine the frequency of *ret* oncogene activation, we analyzed 286 cases of human thyroid tumors of diverse histologic types. We found the presence of an activated form of the *ret* oncogene in 33 (19%) of 177 papillary carcinomas. By contrast, none of the other 109 thyroid tumors, which included 37 follicular, 15 anaplastic, and 18 medullary carcinomas, and 34 benign lesions, showed *ret* activation. (*J. Clin. Invest.* 1992. 89:1517-1522.) Key words: carcinoma • gene rearrangement • *ret* protooncogene • tyrosine kinase • PTC

Introduction

There is growing evidence that tumor development and progression may result from a series of genetic alterations that affect the normal mechanisms controlling cell proliferation (1). Molecular events that have been identified include inactivation of tumor-suppressor genes (2), as well as activating mutations of cellular protooncogenes (3). In some cases, specific oncogenes are altered in specific tumors. Thus, the *N-myc* and *c-erb-B2/neu* oncogenes are amplified in neuroblastomas (4) and in breast carcinomas (5), respectively. Similarly, *c-myc* and *c-abl* are involved in the chromosomal translocations which characterize Burkitt's lymphoma and chronic myelogenous leukemia (3), respectively. Moreover, there is some evidence suggesting a specificity for activation of particular *ras* gene family members in some forms of cancer: e.g., *K-ras* in lung, colon, and pancreas carcinomas (6-8), and *N-ras* in acute myeloid leukemia (9).

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The thyroid provides an attractive model to study the steps that are involved in the neoplastic process. Most thyroid neoplasms originate from a single cell type, the thyroid follicular cell. However, they comprise a broad spectrum of tumors with different phenotypic characteristics and variable biological and clinical behavior: i.e., from the benign colloid adenomas, through the slowly progressive differentiated papillary and follicular carcinomas, to the invariably fatal anaplastic carcinomas (10). Papillary and follicular carcinomas are considered to be two different biological entities. Typically, papillary carcinoma is multifocal. It generally metastasizes to regional lymph nodes, and has been associated with previous radiation exposure. Conversely, follicular carcinoma is solitary and encapsulated. It invades blood vessels and spreads often to bones; its highest incidence has been reported from endemic goitrous areas (10). It seems probable that the differences observed in biological behavior could be explained by the involvement of different genetic events in the pathogenesis of these two differing follicular cell-derived malignancies.

We have recently demonstrated the frequent activation of a new oncogene in human papillary thyroid carcinomas (11-13). We have shown that this oncogene, named papillary thyroid carcinoma (PTC),¹ is the product of the recombination of the tyrosine-kinase domain of the *ret* protooncogene with a thus far uncharacterized gene that we have named H4 (13). In addition, we have observed that an inversion (10)(q11.2-q21) is present in a significant number of papillary thyroid carcinomas (14). The *ret* and H4 genes are both localized on the long arm of the chromosome 10 (15-17), and we have demonstrated that at least in four cases, the inversion on chromosome 10 determines their fusion (17a).

To confirm the frequency of activation of *ret* in papillary tumors in a wider collection of samples, and to ascertain what role *ret* activation may play in the pathogenesis of nonpapillary thyroid tumors, we have analyzed 286 human thyroid neoplasms collected at the Mayo Clinic, Rochester, Minnesota, at the Hospitals of Lyon, France, at the Istituto Nazionale Tumori of Milan, and at the II Medical School of Naples, Italy. In this study we demonstrate that *ret* oncogene is frequently activated only in the papillary histotype of thyroid carcinomas. From these findings, we would suggest that *ret* activation may represent a specific genetic event that may be of value in the differential diagnosis of papillary thyroid cancer in man.

1. Abbreviations used in this paper: PCR, polymerase chain reaction; PTC, papillary thyroid carcinoma.

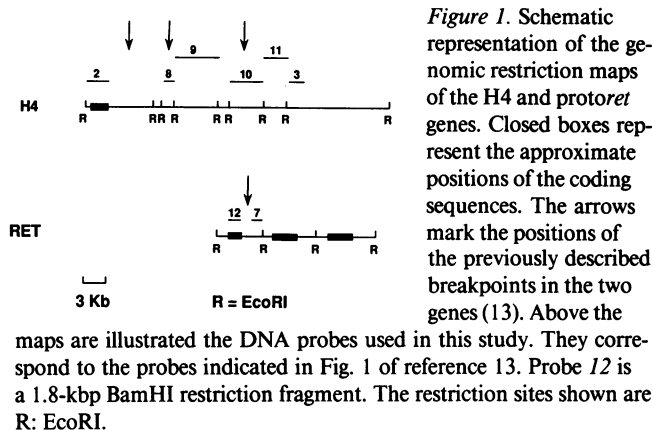


Figure 1. Schematic representation of the genomic restriction maps of the H4 and protoret genes. Closed boxes represent the approximate positions of the coding sequences. The arrows mark the positions of the previously described breakpoints in the two genes (13). Above the

maps are illustrated the DNA probes used in this study. They correspond to the probes indicated in Fig. 1 of reference 13. Probe 12 is a 1.8-kbp BamHI restriction fragment. The restriction sites shown are R: EcoRI.

Methods

DNA extraction and Southern blot analysis. The tumor samples were frozen in liquid nitrogen and stored frozen until DNA extractions were performed. The specimens obtained from the Mayo Clinic were evaluated for their content in tumoral cells. A certain degree of contamination by normal cells was observed. However, DNA extractions were performed from those sections containing not less than 70% of neoplastic cells. High molecular weight DNA extraction from tumors and Southern blot analyses were performed, according to standard procedures (18). Briefly, 10 μ g of DNA were digested, as recommended by the enzyme manufacturer (Amersham Corp., Arlington Heights, IL; Promega Biotec., Madison, WI), electrophoresed through 0.8% agarose, transferred to nylon filters (Hybond-N; Amersham Corp.), and hybridized to 32 P-labeled probes by the random oligonucleotide primer kit (Amersham Corp.). Hybridization and washings were carried out

under stringent conditions, as previously described (13). Autoradiography was performed by using Kodak XAR films at -70°C for 1–7 d with intensifying screens.

Transfection assay. The DNAs extracted from tumor specimens were transfected onto NIH/3T3 cells by the calcium phosphate coprecipitation technique, following a modified version of the protocol of Chen and Okayama, as previously described (12). Foci of transformed cells were evaluated after 2–3 wk.

RNA analysis and polymerase chain reaction (PCR). RNA extraction was performed, as previously described (19). The RNA amplification was done following the method described by E. S. Kawasaki (20), starting from 5 μ g of total RNA. The sequences of the sense H4-(196–213) and the antisense *RET* (544–561)-related primers were as follows: H4: 5'-ATTGTCATCTCGCCGTTTC-3'; *RET*: 5'-CTGCTTCAG-GACGTTGAA-3'. The break-point is at nucleotides 351–352. The expected amplified product of the PTC mRNA is 363 bp long.

Results

Southern blot analysis. The *ret* protooncogene encodes a protein structurally related to transmembrane receptors with a cytoplasmic tyrosine-kinase domain (21–23). We have demonstrated that the truncation of the NH_2 -terminal region leads to its activation in thyroid papillary carcinomas. This rearrangement occurs in an intronic sequence that lies between the TK and the transmembrane-encoding domain of the *ret* protooncogene, and it is possible to detect this rearrangement by Southern blot analysis of the tumor DNA (13). Fig. 1 shows a schematic representation of the genomic restriction maps of *ret* and H4, and of the rearrangements between the two genes previously reported (13).

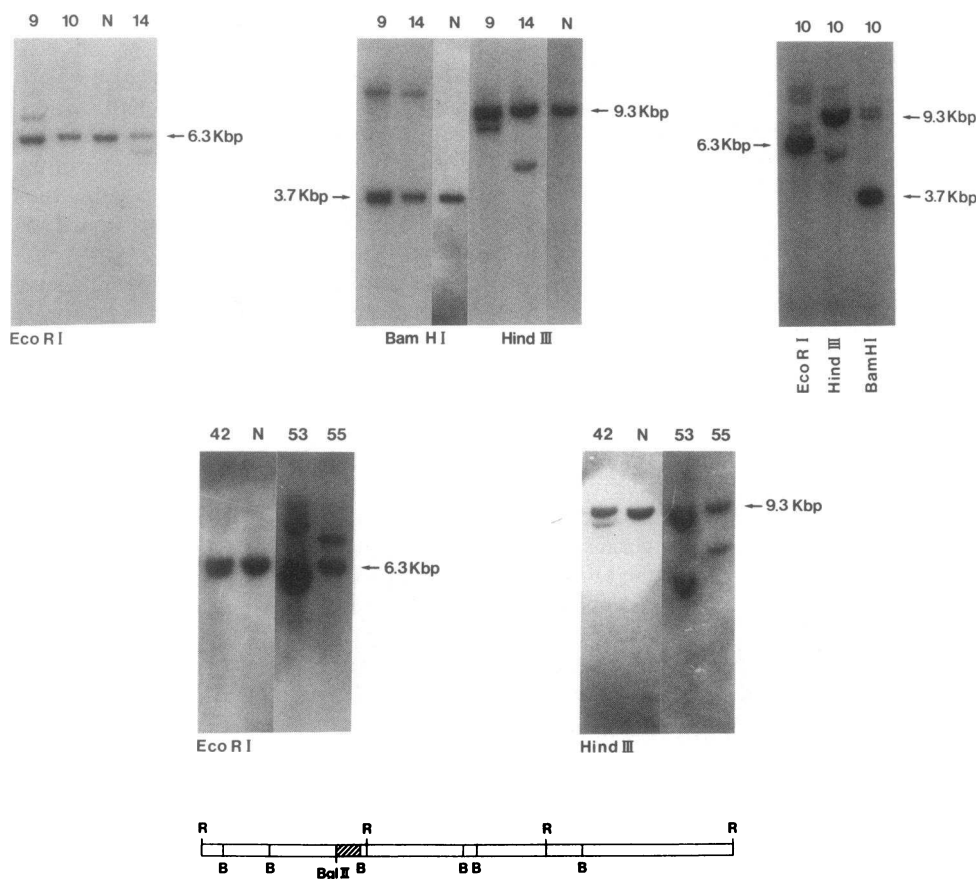


Figure 2. Examples of *ret* rearrangement in papillary thyroid carcinomas collected at the Hospitals of Lyon. Southern blot analysis of DNA extracted from the neoplastic specimens. 10 μ g of DNA were digested with EcoRI, BamHI, or HindIII restriction enzymes (Amersham Corp.), transferred to nylon filters (Hybond-N; Amersham Corp.) and hybridized to a 1.0-kbp BamHI-BglII *ret*-specific probe. The arrows indicate the size of the normal fragments. (Lanes 9, 10, 14, 42, 53, and 55) DNAs extracted from different tumoral samples. (Lane N) DNA extracted from normal human tissue. A partial restriction map of the region of the *ret* gene analyzed and the probe used (shaded area) are shown.

Here, we report the analysis of 286 neoplastic thyroid samples for *ret* activation. This analysis has been performed by probing Southern blots with a 1-kbp BglII-BamHI DNA fragment. This fragment is able to identify the region within the *ret* gene where rearrangement occurs (Probe 7 of Fig. 1) (13). This probe hybridizes to a 6.3-kbp EcoRI, a 3.7-kbp BamHI, and a 9.3-kbp HindIII restriction fragment in normal human DNA (see map on Fig. 2). We found that 33 (11.5%) out of 286 samples showed adjunctive rearranged bands, and this result was demonstrable with at least three different restriction enzymes (examples are in Figs. 2 and 3). In some cases, the rearranged band had a decreased intensity with respect to the normal one (for example, lane 10, Fig. 2). This is most likely due to contamination of the tumoral specimen by normal surrounding tissue. The possibility that the rearrangement might involve only a fraction of the neoplastic cells is currently under investigation by using the in situ RNA hybridization technique.

The rearrangement was specific for the papillary histotype; it was present in 11 out of 65 papillary samples (16.9%) collected at the Mayo Clinic, 8 out of 70 (11.4%) from Lyon, and 14 out of 42 (33.3%) from Milan. We did not find any rearrangement in the 37 follicular, 15 anaplastic, and 18 medullary carcinomas, nor in the 34 benign lesions that we have analyzed (Table I).

Transfection assay. We evaluated the transforming activity of the 14 samples collected at the Istituto Nazionale Tumori of Milan which already scored positive for *ret* rearrangement. All

Table I. *RET* Activation in Human Thyroid Tumors

Tumor type	Positive/analyzed			
	USA	France	Italy	Total
Papillary	11/65	8/70	14/42	33/177
Follicular	0/11	0/13	0/13	0/37
Anaplastic	0/2	0/5	0/8	0/15
Medullary	0/0	0/3	0/15	0/18
Adenoma	0/0	0/18	0/16	0/34
*Other	0/0	0/1	0/4	0/5
Total	78	110	98	286

* Squamous cell carcinoma, sarcomatoid carcinoma.

the samples, tested by the standard focus formation assay on NIH/3T3 cells, gave rise to transformed foci in 2–3 wk (Table II). Also, the available metastases of the *ret*-positive tumors scored positive (Table II). Hybridization of the DNA extracted from the primary foci of transformed NIH/3T3 cells with *ret*-specific probes allowed us to conclude that the transferred oncogene was actually an activated version of *ret* (data not shown). We also transfected the DNA extracted from nine follicular tumors; none gave rise to transformed foci (Table II).

Further characterization of gene rearrangement. We had previously reported that when tumors carrying an activated version of *ret* were probed with a NH₂-terminal *protoret*-speci-

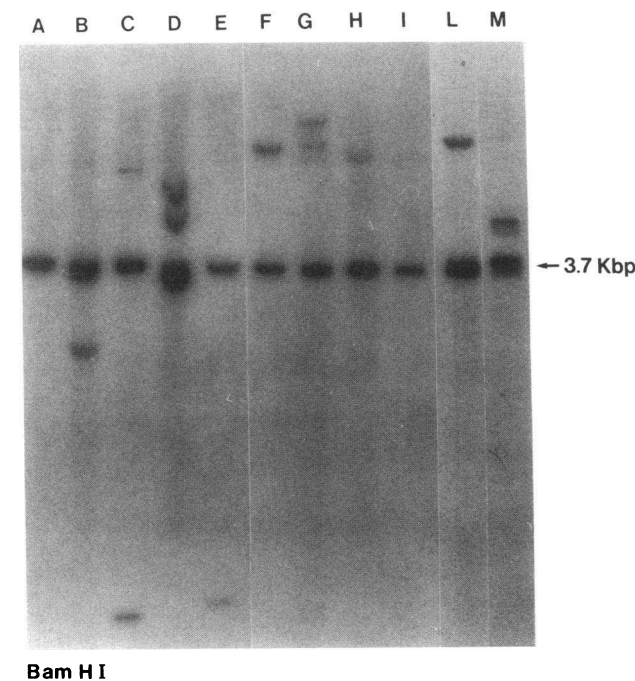


Figure 3. Examples of *ret* rearrangement in papillary thyroid carcinomas collected at the Mayo Clinic. (Lanes B, C, D, E, F, G, H, L, and M) DNAs extracted from different tumoral samples. (Lanes A and I) DNA extracted from normal human tissue.

Table II. Transforming Activity of the Activated *RET* Oncogene from Papillary and Follicular Thyroid Carcinomas

Patient No.	Histological type	Specimen*	Transforming activity [†]	H4 positivity
1	Papillary Ca.	T	0.05	yes
1		M	0.06	yes
2		T	1.10	yes
2		M	0.03	yes
3		T	0.10	no
3		M	0.30	no
4		T	0.20	yes
4		M	0.03	yes
5		T	0.07	no
5		M	0.10	no
6		M	0.05	no
7		T	0.35	no
8		T	0.15	yes
9		T	0.63	no
9	M	0.85	no	
10	T	0.31	yes	
11	M	0.65	yes	
12	T	0.08	no	
12	M	0.75	no	
13	M	0.07	yes	
14	T	0.15	no	
15–24	Follicular Ca.	T	<0.003	no

* T, Primary tumor; M, Lymph nodal metastasis.

[†] NIH/3T3 Transformed foci/ μ g of DNA transfected.

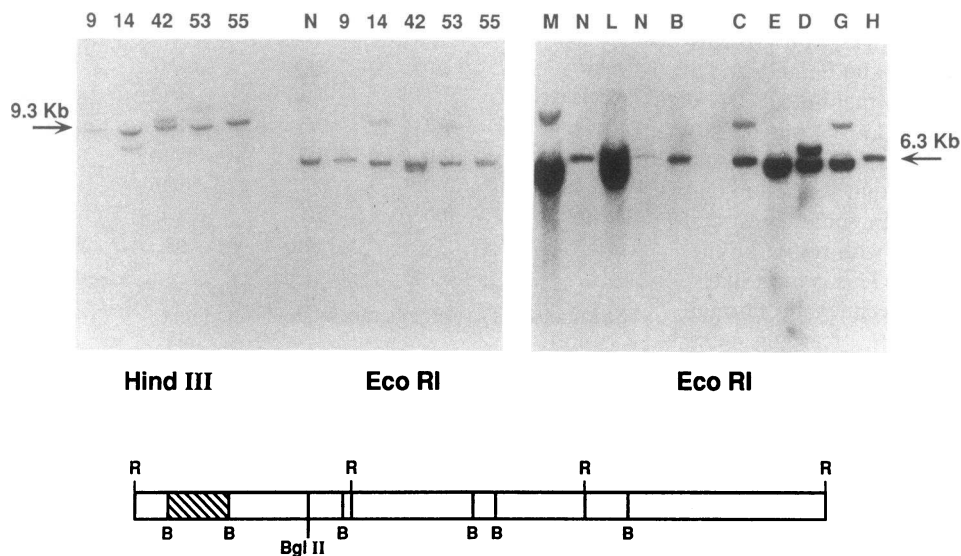


Figure 4. Rearrangement of the *ret* 5' terminal region in papillary thyroid carcinomas. Southern blot analysis of DNA extracted from the neoplastic specimens. 10 μ g of DNA were hybridized to a 1.8-kbp DNA probe specific for the 5' terminal region of the *ret* protooncogene. The arrows indicate the size of the normal fragment. A partial restriction map of the region of the *ret* gene analyzed and the probe used (shaded area) are shown. (Left) Samples collected at the Hospitals of Lyon. (Lanes 9, 10, 14, 42, 53, and 55) DNAs extracted from different tumoral samples. Lane N: DNA extracted from normal human tissue. (Right) Samples collected at the Mayo Clinic. (Lanes B, C, D, E, G, H, L, and M) DNAs extracted from different tumoral samples. (Lane N) DNA extracted from normal human tissue.

fic sequence, a rearranged band was also detectable. This suggests that the chromosomal event leading to *ret* activation should not involve deletion of its NH₂-terminal encoding domain (13). Subsequently, we have demonstrated that an inversion (10) (q11.2-21) determined the H4-*ret* fusion in at least four cases of papillary thyroid carcinoma (17a). In the present study, we found that many of the samples also showed a similar mechanism. In fact, 10/11 samples from Mayo, 5/8 from Lyon, and 9/9 from Milan showed rearranged bands when probed with a 5'-terminal 1.8-kbp *ret*-specific BamHI DNA fragment (probe 12 of Fig. 1) (Examples are shown in Fig. 4).

The PTC oncogene that we had isolated from papillary thyroid carcinomas by means of the transfection assay was shown to be the product of a fusion between a truncated *ret* gene and a novel sequence that we named H4 (13). We demonstrated that the truncation of the H4 gene could occur in different points over a sequence of several thousand base pairs (Fig. 1) (13). Thus, the analysis of H4 involvement has been performed by using different probes (shown in Fig. 1) from Alu-free regions of the H4 gene (probes 2, 8, 9, 10, 11, 3, of Fig. 1) (13). Fig. 5 shows the H4 gene rearrangement in the sample 14 from Lyon (probe 2 of Fig. 1) (13). We have shown H4 rearrangement also in one other sample that was collected at the Mayo Clinic (sample D of Fig. 2) and cytogenetically showed a chromosome 10q inversion (14, 17a). Moreover, 7 out of the 14 samples which scored positive in the transfection assay showed H4 involvement, since in these cases the primary NIH/3T3 foci contained H4-specific sequences (Table II).

RNA and PCR analysis. We have previously shown that *ret* rearrangement leading to PTC formation gives rise to truncated transcripts. The H4 promoter, which fuses to *ret*, is able to drive its expression in the thyroid tumor that carries the rearrangement. To increase the possibility of detecting the PTC formation, and to verify H4 involvement in the cases in which apparently by the Southern blot analysis H4 was not rearranged, we performed a PCR analysis on the RNA extracted from five *ret*-rearranged tumors. We copied 5 μ g of

tumoral RNA by using reverse transcriptase and an antisense 18-bases long 3' *ret*-specific oligonucleotide primer. We amplified the resultant cDNA with the same 3' primer and a sense H4-specific 18-bases oligomer. An amplified 363-bp long fragment indicated the presence of a fused H4-*ret* transcript (i.e., activated PTC). The correct sized fragment was found in samples 10, 14, and 53 from Lyon, but not in samples 9 (Fig. 6) and 55 (not shown). Thus, in samples 10, 14, and 53, *ret* was fused to H4; conversely in the samples 9 and 55, the rearrangement of the *ret* protooncogene probably involved a gene distinct from H4.

Discussion

The identification of the nature of the genetic mutations in thyroid neoplasms, and the assessment of their prevalence in the various tumor phenotypes, is critical to an understanding

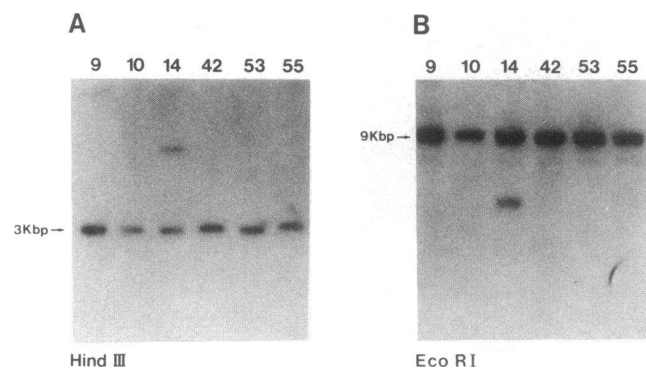


Figure 5. Rearrangement of H4 gene in one papillary thyroid carcinoma. Southern blot analysis of the DNAs extracted from the samples collected at the Hospitals of Lyon. The H4-specific DNA probe used is the probe 2 of reference 13. The arrows indicate the sizes of the normal fragments.

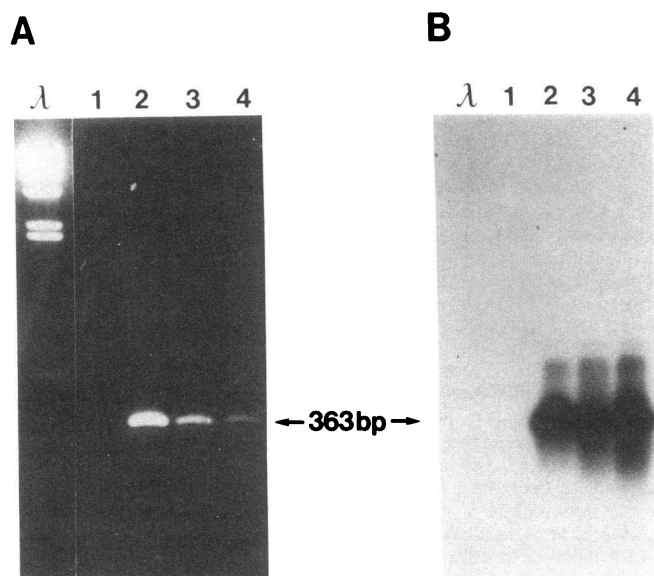


Figure 6. Expression in papillary thyroid carcinomas of a fused H4-*ret* transcript. PCR analysis on the RNA extracted from four papillary carcinomas collected at the Hospitals of Lyon. 5 μ g of total RNA were reverse transcribed using an antisense *ret*-specific oligonucleotide, and amplified using the same oligo together with an H4-specific primer. 1/10 of the product of the amplification was subjected to electrophoretic separation on a 2% agarose gel. (Lanes 2, 3, and 4) RNA extracted from the patients 10, 14, and 53 of Fig. 1, respectively. (Lane 1) RNA extracted from patient 9. (A) Ethidium bromide staining of the agarose gel. (B) Hybridization of the amplified product to a *ret*-specific cDNA probe (18).

of their pathogenesis. Different genetic events may occur to determine the different phenotypic characteristics of thyroid neoplasms, such as the ability to invade the lymphatics (papillary) or the blood vessels (follicular), or the propensity to become dedifferentiated (anaplastic).

We previously demonstrated the activation of the *ret* oncogene in papillary carcinomas (12, 13). To date, the *ret*-transforming gene has been found activated in vivo only in thyroid carcinomas (11–13) and in a papillary thyroid carcinoma cell line (24). Previously described rearrangements of the *ret* oncogene were found to have occurred in vitro during the transfection procedure (25–27). Moreover, we did not find any example of *ret* rearrangement by screening about 150 nonthyroid tumors (data not shown), and Ishizaka et al. have reported a similar result in a study of more than 100 nonthyroid tumors (28).

These published observations suggest that *ret* activation is specifically involved only in thyroid tumorigenesis, but they do not determine if a particular subtype of thyroid tumor has *ret* rearrangements. Therefore, we collected 286 human thyroid neoplastic samples and screened them by Southern blot analysis for *ret* rearrangement. We found *ret* activated only in the papillary subtype of these tumors: specifically, in 11 tumors collected at the Mayo Clinic, 8 collected at the Hospitals of Lyon, and 14 collected in Italy at the Istituto Nazionale Tumori of Milan and at the II Medical School of Naples. Our data strongly support the hypothesis that *ret* activation is specific for papillary carcinoma. In fact, none of either the 37 follicular carcinomas or the 72 other nonpapillary thyroid tumors scored positively.

Recently, Ishizaka et al. (29) published the detection of an activated *ret* (PTC) in 1 out of 11 thyroid papillary carcinomas, but also in 4 out of 19 follicular adenomas and in one of two adenomatous goiters. In the adenomas, however, the activation of *ret* was detected only in some regions of the tumors. They do not report any analysis of nonpapillary malignant tumors. Since the prevalence of occult thyroid carcinomas in the Japanese population is high, it is possible that the PTC-positive cases of follicular adenomas and adenomatous goiter could reflect a minor population of cancerous cells, as the authors suggest in their discussion.

We also noted a difference in the frequency of *ret* activation in different geographic areas, ranging from 11% in the French samples to 33% in the Italian samples. The U. S. tumors had an intermediate frequency of 17%. This may suggest the involvement of different genetic or environmental factors in these three areas. The recent finding of high rates of *ras* codon 61 mutation in thyroid tumors in an iodide-deficient area (30), and the differences detected in the patterns of *ras* mutation between radiation-associated and spontaneous human thyroid carcinomas (31) seem to support such suggestion. It is noteworthy that only one of the patients positive for *ret* activation in this study, had a prior documented radiation exposure.

We have also been able to demonstrate that the H4 gene was involved in *ret* activation in 11 out of 33 *ret* rearranged samples. We demonstrated this involvement in two cases by Southern blot analysis (samples 14 and D), in three cases by PCR analysis (samples 10, 53, and again, 14), and in seven cases (Milan samples) by the transfection assay. We cannot exclude, however, that H4 may be involved in the remaining cases because we did not have enough RNA and DNA to perform PCR or transfection assays, respectively, in the apparently negative samples. In at least 5 of these 22 cases we have conclusively demonstrated that *ret* is not fused to H4, and still other uncharacterized genes may be involved in the rearrangement. Interestingly, cytogenetic studies of papillary carcinoma have demonstrated that the region where *ret* is mapped (10q11.2) is also involved in translocations with other chromosomes (14).

In 24 of 33 *ret*-rearranged cases, the 5' terminal region of the *ret* gene (extracellular and transmembrane encoding region) was not deleted, but was present rather in a rearranged status, similar to previously described cases (13).

There still remain major unanswered questions about the manner in which thyroid tumors evolve, and the molecular events that determine whether a tumor behaves either as a differentiated papillary/follicular or as a dedifferentiated anaplastic one. Other genetic alterations are frequently detected in thyroid tumors and include mutations involving *ras* genes. However, the published studies on *ras* oncogene involvement (30–36) indicate a significant heterogeneity among the results obtained by different groups. Whereas *ras* point mutations are present with an about equivalent frequency in both benign and malignant thyroid tumors, the same conclusion is not readily established when the follicular and papillary types are compared. We have previously demonstrated that the oncogene *trk*, belonging to the tyrosine kinase family, is also frequently (about 10%) activated in papillary carcinomas (12), and recently, Suarez et al. (37) observed *gsp* mutations in ~ 10% of differentiated thyroid carcinomas. In addition, allelic loss studies of chromosome 3 and 10 have demonstrated differences between papillary and follicular carcinomas (38). These data

indicate that other somatic mutations should be considered to explain the differing behavior patterns exhibited by different thyroid tumor types. However, from our studies we would suggest that *ret* activation represents an important and apparently specific genetic event in the development of human papillary thyroid carcinoma.

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