Assessment of RET/PTC Oncogene Activation and Clonality in Thyroid Nodules with Incomplete Morphological Evidence of Papillary Carcinoma

A Search for the Early Precursors of Papillary Cancer

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Noninvasive thyroid nodules that exhibit borderline morphological signs of papillary cancer are difficult to diagnose and we do not know if they represent papillary carcinoma precursor lesions. Forty-six such nodules were analyzed for RET activation by immunohistochemistry and, in selected cases, by reverse transcriptase-polymerase chain reaction performed on RNA extracted after laser capture microdissection (LCM) of the tumor foci with and without papillary carcinoma features and positive RET immunoreactivity. RET immunoreactivity was identified, at least focally, in 30 of 46 (65.2%) of the nodules where it closely paralleled the morphological changes. Enough RNA was obtained after LCM in seven samples. RET/PTC1 or RET/PTC3 were detected in microscopic foci with papillary carcinoma features in most of the thyroid nodules (five of seven cases). No RET/PTC1 or RET/PTC3 rearrangements were detected in areas of the same tumors that lacked the cytological alterations. Analysis of clonality in the same nodules selected for LCM demonstrated that two were monoclonal and six were polyclonal. We conclude that RET activation closely parallels the morphological changes, that it is restricted to those areas of the tumor with the cytological alterations and that it is detectable in both mono- and polyclonal tumors. Although the finding of microscopic foci indicative of papillary carcinoma in a hyperplastic or adenomatous nodule does not justify the interpretation of the entire lesion as papillary carcinoma, it is possible that such foci may precede the development of invasive papillary cancer. (Am J Pathol 2002, 160:2157–2167)

Thyroid nodules are clinically evident in ~5% of women and 1% of men and therefore represent a very common type of endocrine pathology in humans. The vast majority of them (>90%) are benign, ie, either hyperplastic nodules or follicular adenomas. When malignant, they are usually examples of papillary thyroid carcinoma. However, the histological diagnosis of well-circumscribed thyroid nodules without capsular or vascular invasion is not always straightforward. As a matter of fact, sometimes the pathologist has to face the dilemma of whether a noninvasive nodule with minor or incomplete microscopic signs of papillary carcinoma should be classified as malignant or not. Understanding the biology of such tumors is difficult, also considering the fact that premalignant lesions of the thyroid (or, for that matter, those of endocrine glands in general) are unknown or poorly defined. Rearranged versions of the RET proto-oncogene called RET/PTC (for papillary thyroid carcinoma) are a marker for papillary thyroid cancer. RET/PTC results from the fusion of the RET tyrosine-kinase (TK) domain with the 5'-terminal region of heterologous genes, which leads to the formation of RET chimeric oncogenes. To date, at least 15 such chimeric mRNAs involving 10 different genes have been reported, of which RET/PTC1 (resulting...
from the fusion of RET with the H4 gene) and RET/PTC3 (resulting from the fusion of RET with the RFG gene) are by far the most common. It is not known whether the cytological alterations observed in thyroid tumors with borderline morphological features of malignancy reflect RET/PTC activation nor is it clear whether they occur as part of a clonal proliferation of thyroid epithelial cells. To address these issues and to better understand the process of thyroid tumorigenesis we have analyzed RET activation and clonality in 46 noninvasive thyroid nodules with minimal or incomplete evidence of papillary carcinoma according to the flow chart illustrated in Figure 1. The cases were studied by immunohistochemistry with RET(TK) antibodies and by reverse transcriptase-polymerase chain reaction (RT-PCR) for RET/PTC1 and RET/PTC3 performed on RNA extracted after laser capture microdissection (LCM) of tumor foci with and without papillary thyroid carcinoma nuclear changes and RET(TK) immunoreactivity selected for LCM were specifically included and material dissected from both foci was submitted together for DNA extraction.

Materials and Methods

Tumor Samples and Histopathological Classification

The surgical pathology files of Yale-New Haven Hospital from 1985 to 2001 were searched for cases in which the terms “focal papillary carcinoma” and either “nodular hyperplasia” or “follicular adenoma” coexisted in the diagnostic field of the pathology report. A similar search was performed in the personal consult files of one of the authors (JR). After review of the search results, 46 thyroid nodules with minimal or incomplete evidence of papillary carcinoma were selected for the study; 34 cases originating from Yale-New Haven Hospital and 12 from the consult files. The former represented 2.5% of all thyroidectomy specimens diagnosed in the Surgical Pathology Laboratory of Yale-New Haven Hospital during the 1985 to 2001 period. Case selection was based on the histological findings and on the availability of adequate diagnostic material (including the paraffin blocks) and clinicopathological data. All microscopic sections from these 46 cases were reviewed. For the purpose of the study, the thyroid nodules were classified into one of the four categories illustrated in Figure 2 according to the terminology recommended by Rosai and colleagues. The cytological signs of papillary carcinoma considered in this study were clearing, overlapping, and irregularities of the nuclear contour in the form of indentations, grooves, and pseudoinclusions. The overall degree of the cytological alterations was estimated in each case and described as “poorly” or “fully” developed. Histological changes were considered focal when they involved <10% and widespread when they involved at least 53% of the thyroid nodule.

Figure 1. RET activation was analyzed in 46 thyroid nodules by immunohistochemistry with RET(TK) antibodies. In selected cases positive by immunohistochemistry, RT-PCR for RET/PTC1 and RET/PTC3 was performed on RNA extracted after LCM of the tumor foci with and without papillary thyroid carcinoma nuclear changes and RET(TK) immunoreactivity. Clonality was analyzed by PCR using the polymorphic human androgen receptor gene (HUMARA) as marker for chromosome X inactivation. Areas of the nodule corresponding to the foci with and without papillary thyroid carcinoma nuclear changes and RET(TK) immunoreactivity selected for LCM were specifically included and material dissected from both foci was submitted together for DNA extraction.

Figure 2. Thyroid nodules with incomplete morphological evidence of papillary carcinoma but no vascular or capsular invasion were classified into one of four categories according to the terminology recommended by Rosai and colleagues. The cytological signs of papillary carcinoma considered in this study were clearing, overlapping, and irregularities of the nuclear contour in the form of indentations, grooves, and pseudoinclusions. The overall degree of the cytological alterations was estimated in each case and described as “poorly” or “fully” developed. Histological changes were considered focal when they involved <10% and widespread when they involved at least 53% of the thyroid nodule.

RET Activation

Antibodies and Immunohistochemistry

Polyclonal rabbit antibodies were raised against the tyrosine kinase domain of human RET expressed as recombinant glutathione S-transferase fusion protein. They
were affinity-purified by sequential chromatography first on RET and then on GST-coupled agarose columns. The RET(TK) antibodies react with both full-length and rearranged RET in RET/PTC. The specificity of the RET(TK) antibodies used in this study was tested by immunoblotting of protein lysates obtained from NIH3T3 cells transfected with RET/PTC1 and RET/PTC3 oncogenes.9,10 Protein extractions and immunoblotting were performed according to standard procedures and immune complexes were detected by the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The reliability of this RET(TK) antibody for immunohistochemistry has been verified in a recent study.11 Formalin-fixed, paraffin-embedded 4-μm-thick histology sections were obtained from representative blocks for each of the 46 nodules. Immunohistochemistry was performed according to established protocols using a 1/200 dilution of the RET(TK) antibody and the DAKO Envision kit (DAKO, Carpinteria, CA). Negative controls were performed on all cases by omitting the primary antibody. Sections of medullary thyroid carcinoma12 and of previously characterized papillary thyroid carcinomas13 were used as positive controls. Positive immunoreactivity for anti-RET(TK) was abolished by preadsorption with a molar excess of the RET protein. To allow for direct comparison of the immunohistochemical results, sections were cut from paraffin blocks that included areas with and without FTC-NC. Cases were scored as positive when distinct brown staining was observed in the epithelial cells of the thyroid nodules.

**LCM and RNA Extraction**

Fourteen cases with discrete foci of positive RET(TK) immunoreactivity, abundant lesional material and optimal tissue preservation were further processed for RNA extraction and nested RT-PCR after LCM (Figure 1) following the general procedures outlined at the National Institute of Health LCM web site (http://dir.nichd.nih.gov/lcm/lcm.htm). Serial 5-μm sections corresponding to those stained for immunohistochemistry were mounted on plain nonadhesive glass slides, deparaffinized, and stained with methyl green. The microtome and the water bath were decontaminated before cutting in each case. The number of serial sections cut per case ranged from four to eight depending of the size of the RET(TK)-positive foci within the thyroid nodules that were identified in the methyl green-stained sections and targeted for LCM. Also targeted for LCM were the corresponding RET(TK)-negative areas in the same thyroid nodule. LCM was performed using a PixCell I system (Arcturus Engineering, Mountain View, CA). Approximately 1000 30-μm shots were used to transfer on the thermoplastic film-coated cap cells obtained from each thyroid nodule. RNA was extracted according to established protocols.14 Briefly, each cap was placed in an Eppendorf tube containing 200 μl of 6 mg/ml Proteinase K (Sigma Chemical, St. Louis MO), 1 mol/L guanidinium thiocyanate, 25 mmol/L β-mercaptoethanol, 0.5% Sarkosyl, 20 mmol/L Tris-HCl, pH 7.5. The Eppendorf was inverted multiple times to fully digest the tissue off the cap. Twenty μl (0.1× volume) of 2 mol/L sodium acetate, pH 4.0, and 220 μl (1× volume) of water-saturated phenol were added to the RNA extraction solution followed by chloroform-isooamyl alcohol (0.3× volume). After vigorous vortexing and cooling on wet ice the samples were centrifuged to separate the aqueous and organic phases. The aqueous phase was transferred to a new tube containing 1 μl of glycerol solution (10 μg/ml) used as a carrier and to facilitate pellet visualization. After adding an equal volume of cold isopropanol the RNA was precipitated at −20°C overnight, centrifuged, washed with ethanol, treated with DNase, and re-extracted. The pellets were stored at −80°C.

**Nested RT-PCR for RET/PTC1 and RET/PTC3 Rearrangements**

Three μmol/L of resuspended RNA from the 14 cases selected for LCM were reverse-transcribed with 2.5 μmol/L of random hexamers in a 20-μl reaction mix containing 2.5 U/μl murine leukemia virus (MuLV) RT, 5 mmol/L MgCl2, 1 mmol/L each dNTP, and 1 U/μl RNase inhibitor in 1× PCR buffer II (Perkin-Elmer, Foster City, CA). The thermoprofile for cDNA generation was 25°C for 10 minutes, 42°C for 60 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. RT-PCR with primers specific for the human aldolase gene was used for mRNA control. The aldolase + primer was 5′-CGC AGA AGG GTG CCT GGT GA-3′ (nucleotides 18 to 37 of exon 1), the aldolase − primer was 5′-CAG CTC CTT CTT CTG CTC CG-3′ (nucleotides 175 to 194 of exon 2).15 The expected 176-bp product for aldolase was obtained from microdissected material in 7 of the 14 cases and only these were further analyzed. RET/PTC1 and RET/PTC3 transcripts were investigated using nested RT-PCR. The primer sequence and location are shown in Figure 3. For PCR, 3 μl of the cDNA template were used for the first round of amplification with the external primer sets (Figure 3) in a 30-μl reaction volume with 0.1 μmol/L for each primer, 200 μmol/L each dNTP, 0.8 U AmpliTaq DNA polymerase in Buffer II containing 2.0 mmol/L MgCl2 (Perkin-Elmer). After a 12-minute hot start at 94°C, nine cycles of touch-down amplification were performed (progressively lowering the annealing temperature from 61°C to 55°C), followed by 40 cycles of amplification (94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 45 seconds) with a Perkin-Elmer 9700 thermal cycler. For the second round of amplification, 2 μl of first round PCR product were used with the internal primer sets (Figure 3) and the same reaction conditions described for the first amplification round. The nested RT-PCR products for RET/PTC1 and RET/PTC3 were analyzed on a 3% agarose gel and hybridized with a probe covering the tyrosine-kinase domain of RET.14 RNA extracted from previously characterized papillary carcinoma samples15,14 were used as positive controls. Amplification in the absence of RT, or in the presence of RNA extracted from the undifferentiated thyroid carcinoma cell line ARO that lacks RET/PTC rearrangement,16 was used as a negative control.
HUMARA Assay for Clonality Assessment

Of the 14 thyroid nodules selected for LCM and nested RT-PCR, 11 originated in female patients allowing for investigation of the chromosome X inactivation pattern. This was analyzed in the 11 thyroid nodules using a PCR-based assay for the polymorphic human androgen receptor gene (HUMARA). Four serial 10-μm-thick unstained paraffin sections corresponding to those analyzed by immunohistochemistry and used for LCM were mounted on plain glass slides and deparaffinized (Figure 1). Approximately 10 to 20 μg of tissue were manually dissected from the unstained sections of the thyroid nodules mentioned above. Areas of the nodule corresponding to the foci with and without papillary thyroid carcinoma nuclear changes and RET(TK) immunoreactivity selected for LCM were specifically included. Material dissected from the unstained sections of the thyroid nodules was submitted together for DNA extraction. The presence of the lesional material of interest was documented by microscopic examination of the chromosome X inactivation pattern. In all cases, the PTC-NC were superimposed on thyroid nodules that had otherwise benign histological features. All of the nodules were well circumscribed, with a well-defined tumor capsule in the case of the adenomas and a poorly defined or incomplete one in that of the hyperplastic nodules. In all cases, the morphological signs of papillary carcinoma were incomplete, either qualitatively (when the PTC-NC, ie, clearing, overlapping, and irregularities of the nuclear contour in the form of indentations, grooves, and pseudo-inclusions, were not developed enough to ensure an unequivocal diagnosis of papillary carcinoma), and/or quantitatively (when the PTC-NC were not uniformly present throughout the nodule). Those classified in categories A and D accounted for almost 80% of cases. Type D nodules, the single most common category, were characterized by areas with poorly developed PTC-NC present in at least one third of the nodule. Among the cytological changes, nuclear clearing, occasional grooves, and some nuclear overlapping were the most common alterations encountered. Type A nodules, the second most common category, were characterized by the presence of one (11 cases), two (3 cases), or more (2 cases) discrete papillary carcinoma foci in the background of an otherwise benign nodule. The size of the individual papillary carcinoma foci ranged from <0.1 cm to 0.7 cm, whereas the large majority of the thyroid nodules (90% or more) was histologically benign. The overall appearance of the type C nodules was similar to that described for the macrofollicular variant of papillary carcinoma whereas type B nodules had features intermediate between type A and type C nodules. Two cases (both in the type A nodule category) developed in patients who had received radiation in the head and neck region as children. All cases in the A, B, and C categories were diagnosed and treated as well-differentiated thyroid carcinomas. In only one case (type B nodule, Table 1), did the tumor present with metastases to the regional lymph nodes. One patient with a type C nodule died of alcoholic cirrhosis with no evidence of thyroid carcinoma at autopsy. Limited follow-up information in 25 additional
patients does not indicate tumor recurrence after an average follow up of 4.4 years (range, 11 months to 13 years).

**RET(TK) Immunohistochemistry**

Antibody specificity was confirmed by immunoblotting with anti-RET(TK) performed on protein lysates obtained from NIH3T3 cells expressing RET/PTC1 or RET/PTC3 constructs (Figure 4). As illustrated in Figure 1, the thyroid tumors were initially screened for RET expression. Many cases (30 of 46, 65.2%) were positive after immunohistochemistry with RET(TK) antibodies (Table 1 and Figure 5). Positive immunoreactivity was restricted to areas featuring PTC-NC, although the proportion of positive cells varied in such areas according to the different types of thyroid nodules. In general, it was higher (and the staining intensity stronger) in those areas with fully developed PTC-NC, opposed to those with poorly developed nuclear changes. In fact, the two most common thyroid nodule categories (A and D), which also represented the extremes in the spectrum of lesions analyzed in this series (type A nodules having focal but well-developed PTC-NC and type D nodules having widespread but poorly developed PTC-NC) differed in their extent of RET(TK) immunoreactivity: foci with PTC-NC in type A nodules were more often RET(TK)-positive and when positive, expressed RET in a larger proportion of cells compared with type D nodules (Table 1). Among the five type A nodules with two or more PTC-NC foci, RET(TK)-positive cells were present in all of the PTC-NC areas in three cases, whereas no RET(TK) positivity was identified in any of the PTC-NC foci in the remaining two nodules. The presence of papillae within the nodule did not correlate with RET(TK) immunoreactivity, which was only dependent on the presence of the nuclear changes. Also, neither the size nor type of background changes (hyperplastic versus adenomatous) nor the patient’s age or sex influenced RET(TK) immunoreactivity. Seven of the nine cases with mild to moderate lymphocytic thyroiditis surrounding the thyroid nodule were RET(TK)-positive.

**RET/PTC1 and RET/PTC3 Oncogene Activation and Clonality**

A summary of the results for the nine tumors in which either RET/PTC or the HUMARA gene analysis was informative is shown in Table 2. We selected 14 RET(TK)-positive cases to investigate RET/PTC1 or RET/PTC3 after LCM of material from both RET(TK)-positive and RET(TK)-negative areas of the nodule (Figure 5). Amplification of a housekeeping gene (aldolase) was obtained from micro-dissected tissue in 7 of the 14 cases and only these were further analyzed for RET/PTC1 and RET/PTC3 by nested

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**Table 1. Clinicopathological Findings and RET(TK) Immunoreactivity in the Thyroid Nodules with Minimal Papillary Thyroid Carcinoma Features**

| Diagnostic category* | Number of cases | Female sex | Age† | Thyroid nodule type | Thyroid nodule size‡ | Cases with lymphocytic thyroiditis§ | Cases with lymph node metastases | Cases with papillae in areas with PTC-NC¶ | RET(TK) positive cases | RET(TK)-positive cells | Number of cases |
|----------------------|-----------------|------------|------|--------------------|--------------------|-------------------------------|-------------------------------|-------------------------------|----------------------------|-----------------|----------------|----------------|
| A                    | 16              | 12         | 48.2 | Hyperplastic       | n = 11             | Adenomatous                  | 2                             | 6                             | 11                          | >75%            | 4              |                |
|                      |                 |            |      |                    | n = 5               |                              |                               |                               |                              | 25–75%          | 6              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | <25%            | 1              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | 0%              | 5              |                |
| B                    | 6               | 5          | 46.2 | Hyperplastic       | n = 2              | Adenomatous                  | 2.6                           | 1                             | 1                           | >75%            | 5              |                |
|                      |                 |            |      |                    | n = 4               |                              |                               |                               |                              | 25–75%          | 2              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | <25%            | 3              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | 0%              | 1              |                |
| C                    | 4               | 2          | 40.2 | Hyperplastic       | n = 1              | Adenomatous                  | 2.8                           | 1                             | 0                           | >75%            | 4              |                |
|                      |                 |            |      |                    | n = 3               |                              |                               |                               |                              | 25–75%          | 2              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | <25%            | 0              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | 0%              | 0              |                |
| D                    | 20              | 19         | 41.7 | Hyperplastic       | n = 5              | Adenomatous                  | 2.6                           | 5                             | 0                           | >75%            | 10             |                |
|                      |                 |            |      |                    | n = 15              |                              |                               |                               |                              | 25–75%          | 4              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | <25%            | 6              |                |
|                      |                 |            |      |                    |                     |                              |                               |                               |                              | 0%              | 10             |                |

*The diagnostic categories (same as in Figure 2) are: A, focal, well-developed PTC-NC (ie, well-developed PTC-NC involving ≤10% of the thyroid nodule); B, focal, well-developed PTC-NC, rest of the nodule with poorly developed PTC-NC (ie, well-developed PTC-NC involving >10% of the thyroid nodule, rest of the nodule with incomplete PTC-NC); C, widespread, well-developed PTC-NC (ie, well developed PTC-NC involving ≥33% of the thyroid nodule); D, widespread, poorly developed PTC-NC (ie, incomplete PTC-NC involving >33% of the thyroid nodule).

†Average values.

‡Mild (six cases) or moderate (three cases) lymphocytic thyroiditis was present in the thyroid tissue surrounding the thyroid nodule.

§PTC-NC, papillary thyroid carcinoma-type nuclear changes.

¶Positive RET(TK) immunoreactivity was always associated with areas featuring PTC-NC although the proportion of positive cells in such areas varied. RET(TK)-positive cases were divided according to the proportion of positive cells in the areas with PTC-NC in four groups with 0% (negative cases), <25%, 25 to 75%, >75% immunoreactive cells, respectively.
We have analyzed RET activation and clonality in 46 thyroid nodules with minimal or incomplete morphological features of papillary carcinoma. Although there was a rather diverse spectrum of changes in individual tumors, all shared two important features: they were well-circumscribed nodules of difficult diagnostic interpretation. In all cases cytological alterations, which are relevant for the identification of papillary thyroid cancer, were either quantitatively or qualitatively insufficient to ensure an unequivocal pathological diagnosis. As a rule these tumors displayed a predominant or exclusive follicular growth pattern only occasionally admixed with hyperplastic-appearing papillary structures. According to current nosology, nodules with these features should be placed into one of two categories: benign (nodular hyperplasia or follicular adenoma) or malignant (follicular variant papillary carcinoma). Obviously the pathologist's diagnostic decision in dealing with these cases is not simply a matter of tumor classification. Despite the excellent prognosis of well-differentiated thyroid cancer, a malignant diagnosis carries relevant clinical and therapeutic implications as well as psychological consequences for the patient.

This study demonstrates that RET is frequently, albeit focally, expressed in these nodules. In fact, there was at least some degree of RET(TK) immunoreactivity in approximately two thirds of the cases in our series. RET(TK) immunoreactivity was closely associated to the areas within the nodule featuring cytological signs of papillary carcinoma. The correlation between the extent of RET(TK) immunoreactivity and the degree of nuclear changes has recently been commented on by other investigators. Because RET(TK) immunoreactivity in thyroid follicular cells correlates with RET/PTC rearrangement, it would seem that oncogenic RET forms are likely present and should therefore be implicated with the development of these lesions. This was confirmed by the analysis for RET/PTC1 and RET/PTC3 in the RET(TK)-positive foci after selective LCM of tumor material. RET/PTC mRNAs were present in RET(TK)-positive foci with PTC-NC (to include cells with both fully and poorly developed nuclear changes) but not in the areas lacking PTC-NC and RET(TK) immunoreactivity. Interestingly, in a minority of cases, despite amplifiable housekeeping mRNA, RET/PTC1 or RET/PTC3 transcripts could not be detected.

Discussion

Despite the excellent prognosis of well-differentiated thyroid cancer, a malignant diagnosis carries relevant clinical and therapeutic implications as well as psychological consequences for the patient.

This study demonstrates that RET is frequently, albeit focally, expressed in these nodules. In fact, there was at least some degree of RET(TK) immunoreactivity in approximately two thirds of the cases in our series. RET(TK) immunoreactivity was closely associated to the areas within the nodule featuring cytological signs of papillary carcinoma. The correlation between the extent of RET(TK) immunoreactivity and the degree of nuclear changes has recently been commented on by other investigators. Because RET(TK) immunoreactivity in thyroid follicular cells correlates with RET/PTC rearrangement, it would seem that oncogenic RET forms are likely present and should therefore be implicated with the development of these lesions. This was confirmed by the analysis for RET/PTC1 and RET/PTC3 in the RET(TK)-positive foci after selective LCM of tumor material. RET/PTC mRNAs were present in RET(TK)-positive foci with PTC-NC (to include cells with both fully and poorly developed nuclear changes) but not in the areas lacking PTC-NC and RET(TK) immunoreactivity. Interestingly, in a minority of cases, despite amplifiable housekeeping mRNA, RET/PTC1 or RET/PTC3 transcripts could not be detected.

Figure 4. Detection of RET protein expression by NIH3T3 cells transfected with RET/PTC1 and RET/PTC3 constructs. Protein lysates (50 μg) obtained from parental NIH3T3 cells (−), NIH3T3 cells transfected with RET/PTC1, or NIH3T3 cells transfected with RET/PTC3 were immunoblotted with RET(TK) antibodies, as indicated. Filters were stripped and stained with antibodies directed against γ-tubulin to assess equal loading levels.

Figure 5. Histological appearance, immunohistochemical staining with RET(TK) antibodies, and LCM of tissue from case 1 (A1 to A7), case 2 (B1 to B6), and case 4 (C1 to C7) of Table 2. In the low magnification images (A1, B1, C1) the yellow rectangles indicate areas of the tumor with papillary carcinoma features whereas the green rectangles indicate the areas lacking them. The areas with and those without papillary carcinoma features were targeted for LCM and separately processed for RNA extraction. A higher magnification of the foci with papillary carcinoma features is shown in A2, B2, and C2. Cells with cytological alterations of papillary carcinoma were immunohistochemically positive with RET(TK) antibodies (A3, B3, C3; the corresponding negative controls are shown in A4, B4, C4). The nuclear features of papillary carcinoma are fully developed in case 1. They are imperfect in case 2 with RET(TK)-positive cells featuring only some nuclear clearing and partial overlapping (B2 to B4), as well as in case 4 where they show minor degrees of nuclear clearing (C2 to C4) and occasional grooves (C3, arrowhead). The RET(TK)-positive cells with nuclear alterations were positioned for LCM (A5, B5, C5), selectively captured (A6, B6, C6), and visualized on the thermoplastic-coated caps (A7, B6 inset, C7) before being processed for RNA extraction. LCM was similarly performed in areas of the nodule lacking the cytological alterations of papillary carcinoma (not shown).
identified in RET(TK)-positive cells with PTC-NC. This could be explained by less stable or lower RET/PTC mRNA levels compared to the housekeeping gene or by technical reasons related to the fact that the mRNA was obtained from laser microdissected tissue. Furthermore, RET rearrangement forms other than RET/PTC1 and RET/PTC3 may be involved. Because our RET(TK) antibody recognizes both rearranged and wild-type RET, it is also
samples) are shown in the controls (RNA extracted from previously characterized papillary carcinoma.14 Positive features. The RT-PCR products were analyzed on a 3% agarose gel and hybridized after LCM from areas of the same thyroid nodules without papillary carcinoma.21,22

possible that RET(TK)-positive cells with PTC-NC may be expressing unrearranged c-RET. This latter possibility seems intriguing in light of the recent reports suggesting a possible role for c-RET in the development of papillary carcinoma.21,22

In keeping with the variability of the morphological features present within each thyroid nodule, the results of X chromosome composition observed in the majority of our cases using the HUMARA gene test are compatible with multiple clones of thyroid follicular cells. This was true regardless of the quantitative and/or qualitative features of the cytological alterations within the tumor and the extent of RET(TK) immunoreactivity. The presence of multiple clones in a given nodule suggests that cells with rearranged RET represent a distinct subset that may have been present in the tumor since its inception from a small group of precursors with a different chromosome X inactivation pattern. However, RET/PTC is also detectable in restricted foci exhibiting papillary carcinoma features in the minority of our tumors that were monoclonal.

In this case, because RET/PTC expression is confined to discrete portions of a monoclonal nodule, it is hard to believe that the rearrangement was present in the cell of origin, indicating that papillary carcinoma may also develop as a secondary event in subclones originating from a single precursor cell. It is important to note that there is a high correlation between RET rearrangement at the DNA level (as detected by fluorescence in situ hybridization) and the presence of RET/PTC chimeric mRNA detected by RT-PCR.23 This makes it unlikely that the expression pattern of RET/PTC in our tumors is the result of focal promoter activity of the rearranged heterologous genes and that the rearrangement is present but the

Figure 6. Nested RT-PCR for RET/PTC1 and RET/PTC3 of RNA extracted after LCM from areas of the thyroid nodules with papillary carcinoma features. The RT-PCR products were analyzed on a 3% agarose gel and hybridized with a probe covering the tyrosine-kinase domain of RET.15 Positive controls (RNA extracted from previously characterized papillary carcinoma samples) are shown in the lanes and 2. Amplification in the absence of RT (lanes 3 and 4), or in the presence of RNA extracted from the undifferentiated thyroid carcinoma cell line ARO that lacks RET/PTC rearrangement16 (lane 12) was used as a negative control. The sample case numbers are those of Table 2. Nested RT-PCR for RET/PTC1 and RET/PTC3 of RNA extracted after LCM from areas of the same thyroid nodules without papillary carcinoma features was negative for RET/PTC1 and RET/PTC3 (not shown).

Figure 7. Amplification of the human androgen receptor gene (HUMARA) with (+) and without (−) previous enzymatic digestion with HhaI of the DNA extracted from the thyroid nodules. DNA was obtained from tissue manually dissected to include in the same sample both foci with and without papillary carcinoma features. The case numbers are those of Table 2. The HUMARA gene on chromosome X contains restriction sites for the methylation-sensitive HhaI enzyme and highly polymorphic CAG repeats adjacent to the exon 1 of the gene. Because of random chromosome X inactivation due to DNA methylation, polyclonal tissue from informative cases that contain both methylated alleles—alleles that are inactive but PCR amplifiable after HhaI digestion of the DNA template—will give two discrete bands in both + and − lanes after PCR of the HUMARA gene, as seen in the samples from cases 3 to 7. On the other hand, tissue originating from a single progenitor cell (ie, monoclonal tissue) contains only one methylated allele and will therefore give a single band after HhaI digestion and PCR as seen in the − lanes for cases 1 and 2. The additional faint bands observed in the + lanes of samples 1 and 2 are consistent with the presence of polyclonal inflammatory cells or stromal tissue.

Table 2. RET Activation and Clonality in Thyroid Nodules with Minimal or Incomplete Evidence of Papillary Carcinoma

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnostic category†</th>
<th>Thyroid nodule type</th>
<th>Thyroid nodule size</th>
<th>RET(TK) immunoreactive foci</th>
<th>RET/PTC1-3 rearrangement in areas with PTC-NC‡</th>
<th>RET/PTC1-3 rearrangement in areas without PTC-NC</th>
<th>Clonality (HUMARA gene assay)</th>
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<td>1</td>
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<td>Yes</td>
<td>RET/PTC3</td>
<td>No</td>
<td>ND**</td>
</tr>
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<td>Yes</td>
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<td>PC</td>
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<td>No</td>
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<td>PC</td>
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*Cases negative for codons 12, 13, and 61 mutations of K-, H-, N-Ras after PCR/SSCP.
†The diagnostic categories (same as in Figure 2) are: A, focal, well-developed PTC-NC; B, focal, well-developed PTC-NC, rest of the nodule with poorly developed PTC-NC; C, widespread, well-developed PTC-NC; D, widespread, poorly developed PTC-NC.
‡PTC-NC: papillary thyroid carcinoma-type nuclear changes.
¶ND, not done (lack of RNA suitable for RT-PCR).
§MC, monoclonal.
∥PC, polyclonal.
**Male patient.
expression of the rearranged RET forms undetectable. Interestingly, it has been shown that thyroid nodules have a variable clonal composition\textsuperscript{24} and that they are genetically heterogeneous.\textsuperscript{25} The possibility that even papillary thyroid carcinoma may consist of multiple subclones is suggested by the common observation of different RET/PTC forms in the same tumor.\textsuperscript{13,30} In this context we have explored the possibility that other oncogenes in addition to RET may be active and that they may represent a confounding factor responsible for the imperfect development of cytological changes, particularly in nodules with poorly developed PTC-NC. However, single-stranded conformation polymorphism analysis of two tumors with poorly developed PTC-NC (cases 4 and 6 of Table 2) failed to demonstrate K-, H-, or N-Ras mutations (data not shown). However, nowhere near 100% of papillary carcinomas have RET/PTC rearrangement and it is possible that oncogenes other than RET/PTC or Ras are involved.

The central problem in the biological interpretation of the microscopic and molecular findings in these nodules with borderline morphological features for papillary carcinoma is whether they should be regarded as fully benign, as benign but undergoing malignant transformation, or as well-differentiated carcinomas. In the light of our results, it is reasonable to consider cases with well-developed PTC-NC (eg, our type A, B, and C tumors) as papillary carcinoma arising in adenomatous or hyperplastic lesions, no matter how focal the PTC-NC may be within the thyroid nodule. In fact, these cases fulfill both morphological and molecular (aberrant RET/PTC transcripts) criteria for such a diagnosis. The diagnosis of carcinoma, however, should be limited to those areas with PTC-NC, an observation that is clinically relevant because tumor size influences thyroid carcinoma staging.\textsuperscript{26} In this regard RET(TK) immunohistochemistry may be a useful adjunct to validate the morphological findings. Cases with only poorly developed PTC-NC are more difficult to categorize. One may argue that because the molecular markers of papillary carcinoma are there, these lesions should be considered malignant as well. In addition, because RET rearrangement seems to be a clonal (or subclonal) process, the “size” of the clone (ie, the relative proportion of the nodule with the PTC-NC) should probably also be taken into account. On the other hand, the finding of tumor-specific molecular alterations per se, without full support of clinico pathological data, does not necessarily imply malignancy: BCL2/JH rearrangement, a molecular marker of follicular center-cell lymphoma, is indeed detectable in a high proportion of lymphoid tissue samples with follicular hyperplasia.\textsuperscript{27} The difficulties encountered in dealing with these cases have recently been addressed by the Chernobyl Pathologists Group after reviewing the histological findings in many thyroid tumors from patients exposed to the fallout from the Chernobyl nuclear fall-out.\textsuperscript{28} These investigators have proposed the term “well-differentiated tumor of uncertain malignant potential” for well-circumscribed, non-invasive tumors with “questionable papillary carcinoma-type nuclear changes.” Their definition (essentially corresponding to the tumors grouped in the D category of this study) seems justified by the finding of foci of cells with RET/PTC transcripts and in consideration of the fact that we know little about the clinical behavior of the tumors. Our limited follow-up information indicates no instances of recurrence, with only one case with focal papillary carcinoma having lymph node metastases at presentation. A longer and more complete follow-up is certainly necessary to draw definitive conclusions, but our data and those of the literature support the view that an extremely good prognosis is expected.\textsuperscript{28}

The finding of aberrant RET/PTC transcripts in tumors with minimal or incomplete evidence for papillary carcinoma and the fact that at least some of them may be diagnosed according to currently accepted criteria as benign thyroid nodules may explain the reports of rearranged RET forms in follicular adenomas and hyperplastic nodules.\textsuperscript{23,29–31} It is also compatible with the observation that multiple samples from different areas of the same tumor are not always positive for RET/PTC when the tumors are diagnosed as follicular adenoma or nodular hyperplasia (whereas they are consistently positive in tumors diagnosed as papillary carcinoma).\textsuperscript{29} Subtle papillary thyroid carcinoma-type cytological alterations are likely present in many of the “benign” nodules with RET/PTC rearrangement reported in the literature. If this assumption is correct, because radiation-induced thyroid carcinogenesis is mediated by RET/PTC oncogenes and results in invasive papillary cancer,\textsuperscript{32–34} the high proportion of RET/PTC rearrangement in radiation-associated follicular adenomas and hyperplastic nodules (40 to 50%, compared to ~10% in sporadic nodules)\textsuperscript{30,31} suggests that thyroid tumors in which the signs of papillary carcinoma are minimal or incomplete may be regarded as premalignant. Indeed, microscopic foci with enlarged nuclei, grooves and even papillae have been noted in benign thyroid tissue from post-Chernobyl specimens.\textsuperscript{35} The early stages of papillary carcinoma development have remained so far elusive. Occult microcarcinoma can be identified in 5 to 20% of autopsy cases and as an incidental finding in ~5% of surgically removed thyroid specimens.\textsuperscript{4} Similar to the tumors described in this series, it is often associated with RET/PTC rearrangement\textsuperscript{14} and has been proposed as a precursor for clinically evident cancer. However, what we know about its natural history\textsuperscript{4} makes it unlikely that it precedes all cases of papillary cancer. The thyroid nodules analyzed in this study do not fit the conventional definition of papillary microcarcinoma\textsuperscript{4} and it is reasonable to speculate that at least some of our cases represent hitherto unrecognized papillary carcinoma precursors. Indeed, in contrast with most clinically evident papillary carcinomas,\textsuperscript{36} the majority of the thyroid nodules in the present study are polyclonal which is in keeping with their interpretation as preneoplastic lesions. Progression to clinically evident cancer would depend on the acquisition of additional genetic alterations the nature of which is yet to be determined. Gene expression analysis has shown that papillary carcinoma reveals a consistent profile.\textsuperscript{37} Analysis of the specific genes over- or underrepresented in papillary carcinoma may contribute significantly to understanding the
sequence of events associated with the progression of precursor lesions to clinically manifest thyroid carcinoma.

In conclusion, RET activation closely parallels the morphological changes in thyroid nodules where it is restricted to the areas of the tumor featuring the cytological alterations of papillary carcinoma and it is detectable in both poly- and monoclonal tumors. Identification of RET/PTC transcripts only in discrete areas of monoclonal nodules indicates that papillary carcinoma may evolve as a secondary event in subclones within the nodule. Although the finding of microscopic foci indicative of papillary carcinoma in a hyperplastic or adenomatous nodule does not justify the interpretation indicative of papillary carcinoma in a hyperplastic or adenomatous nodule does not justify the interpretation of the entire lesion as papillary carcinoma, it is possible that such foci may precede the development of invasive papillary cancer.

References


