

**Metastatic Solid / Insular Papillary Thyroid Carcinomas in *RET/PTC3*  
Transgenic Mice**

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Running title: *RET/PTC3* in variant thyroid carcinoma

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## Abstract

Our research goal is to better understand the mechanisms controlling the initiation and progression of thyroid diseases. One such disease, papillary thyroid carcinoma (PTC), is the leading endocrine malignancy in the United States. Recently, a family of related fusion proteins, *RET/PTC1-5*, has been implicated in the early stages of PTC. Although all nine members of this family have the *c-RET* proto-oncogene kinase domain in their COOH terminus, little is known about how these genes alter follicular cell biology. Consequently, to answer questions related to the mechanism of the *RET/PTC* fusion protein action, we have devised a molecular genetic strategy to study PTC using a mouse model of thyroid disease. We chose to focus our study on a new member of this fusion oncogene family, namely *RET/PTC3*, which has been implicated in more cases of solid tumor carcinoma (79%) than *RET/PTC1* or *RET/PTC2* and predominates (80%) in radiation-induced thyroid cancer of children. We have generated transgenic mice expressing human *RET/PTC3* exclusively in the thyroid. These mice develop thyroid hyperplasia and solid tumor variants of papillary carcinoma. This new transgenic line will be useful in deciphering the molecular and biological mechanisms that cause PTC and histological variations in humans.

## Introduction

Thyroid carcinoma is the most frequent endocrine tumor in the US with the papillary subtype accounting for at least 80% of these cancers (Kini 1987). Chromosomal translocations were identified in these tumors and breakpoints cloned and sequenced. The common characteristic of these breakpoints included expression of fusion proteins, which contain the carboxy terminal kinase domain of either the *c-RET* or *TRK* gene families (Fusco, Santoro et al. 1995; Santoro, Grieco et al. 1995; Takahashi 1995). The invariant expression of the kinase domain in the *RET* type fusion proteins, collectively called *RET/PTC*, suggest an important role in the transformation of thyroid follicular cells (Fusco, Santoro et al. 1995). Several studies have implicated the role of *RET/PTC* fusion genes early in thyroid carcinogenesis, and multiple independent rearrangements have been detected in the same tumor specimen, suggesting that these gene products promote transformation. ((Fugazzola, Pilotti et al. 1995; Fusco, Santoro et al. 1995; Jossart, Greulich et al. 1995; Klugbauer, Lengfelder et al. 1995; Smanik, Furminger et al. 1995; Takahashi 1995; Fugazzola, Pierotti et al. 1996; Santoro, Chiappetta et al. 1996; Sugg, Zheng et al. 1996; Williams, Rooney et al. 1996; Bongarzone, Butti et al. 1997; Cetta 1997; Komminoth 1997; Kusafuka and Puri 1997; Takahashi 1997; Wirtschafter, Schmidt et al. 1997). In support of this, the *RET/PTC* genes have been implicated in the mitogenesis of thyroid cells through the binding of proteins in cell activation (Jossart, Greulich et al. 1995; Klugbauer, Lengfelder et al. 1995; Fugazzola, Pierotti et al. 1996; Rochefort, Caillou et al. 1996; Nikiforov, Rowland et al. 1997). Furthermore, *RET/PTC1* transgenic mice develop follicular hyperplasia and

microcarcinoma although invasive cancer was not observed (Jhiang, Sagartz et al. 1996; Portella, Salvatore et al. 1996; Santoro, Chiappetta et al. 1996).

The recent analysis of thyroid carcinomas from children exposed to radiation following the Chernobyl disaster has shown a high number of thyroid carcinomas expressing the *RET/PTC3* gene (Fugazzola, Pilotti et al. 1995; Klugbauer, Lengfelder et al. 1995; Fugazzola, Pierotti et al. 1996). Interestingly, these tumors appeared morphologically distinct from other papillary cancers containing a high frequency of “solid variant” type carcinomas rather than the characteristic papillary types commonly observed in other papillary tumors (Nikiforov, Rowland et al. 1997). Thus, to investigate the role of *RET/PTC3* in the solid tumor variant of papillary carcinoma and to develop an animal model of thyroid disease, we generated transgenic mice expressing the human *RET/PTC3* gene under the control of the bovine thyroglobulin promoter (Rocheffort, Caillou et al. 1996). These transgenic mice develop thyroid hyperplasia and solid tumor variants of papillary carcinoma with metastatic spread in selected cases. Immunohistochemical analysis of thyroids from transgenic mice shows expression of the *RET/PTC3* fusion protein in thyroid follicular cells. The new *RET/PTC3* mouse strain will be important for studying the mechanisms controlling thyroid cancer progression and differences in variant morphology.

## Materials and Methods

*Generation of transgenic mice.* Constructs for the generation of transgenic mice were cloned into the pBluescript-II SK<sup>+</sup> vector. Plasmid DNA was purified using Qiagen columns according to manufacture's protocol (Qiagen, Inc., Santa Clarita, CA). A DNA construct was made that contained the bovine thyroglobulin promoter (Rochefort, Caillou et al. 1996), the *RET/PTC3* coding sequence and an SV40 polyadenylation signal. For transgenesis, 2 ug of purified construct DNA was microinjected into zygotes as described (Hogan 1986). Founder animals were developed by the Kimmel Cancer Institute Transgenic Facility and identified by Southern hybridization using *RET/PTC3* specific probe (Ausubel 1995). Founder mice were placed into mating with wild type C57BL/6 and progeny examined for the presence of the transgene by PCR.

*DNA amplification by PCR.* The polymerase chain reaction was performed by adding 5  $\mu$ l of template DNA (100 ng) to a 25  $\mu$ l reaction mixture containing 1x PCR buffer, 0.2 mM dNTPs, 50 pM 3' and 5' 21 mer oligonucleotides (5' primer sequence of TGPRO, GGCCAGAGCCCTAAGGTGGGC; and 3' primer *RET*: GATACAGCCTCTCTTCTCCA) and 2.0 U Taq polymerase (Ausubel 1995). The specimens were then placed into a heated lid thermocycler (Hybaid, Inc.) and subjected to 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 60 seconds.

*RNAse protection analysis (RPA).* Thyroid tissue was removed from transgenic mice and homogenized in 0.5 ml of Cell lysis buffer (4M Guanidinium thiocyanate, 25 mM Sodium citrate, 0.5% Sodium N-lauroylsarcosine, 0.1M 2-mercaptoethanol). Nucleic

acid was extracted using phenol:chloroform (1:1) and ethanol precipitated as described (Ausubel 1995). When necessary, DNA was removed by DNase treatment (Rothstein, Johnson et al. 1993). *RET/PTC3* riboprobe was synthesized from a 525bp cloned fragment of the *RET* gene while the control actin riboprobe was synthesized from a 330 bp vector (Ambion). Riboprobes and thyroid RNA were precipitated together, resuspended in 20  $\mu$ L of hybridization buffer (80% deionized formamide, 100 mM sodium Citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, 1 mM EDTA) and incubated overnight at 42°C. RNA hybrids were digested with 40  $\mu$ g/mL RNase A and 2  $\mu$ g/mL RNase T1 for 30 min at 37°C. RNA was precipitated, resuspended in gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 M EDTA, 0.025% SDS), denatured for 3 minutes at 94°C and run on a 5% denaturing polyacrylamide gel for 1 hour at 250 volts. Gels were exposed to X-ray film for 1-5 minutes.

*Western blot analysis.* Tissues were homogenized and cells lysed in protein extract buffer (30 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% Triton X-100, 100 mM NaCl, 1 mM PMSF) and stored at -70°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were then blocked for 1 hour in TBST buffer (20 mM Tris-HCl, pH 7.6, 135 mM NaCl, 1% Tween-20) and incubated at 4°C overnight with preabsorbed anti-*RET* (diluted 1:1000) or anti-phosphotyrosine (diluted 1:1000) antibodies (Santa Cruz Biotech). Following incubation, the membrane was treated with either horseradish peroxidase-conjugated anti-rabbit IgG (Promega) or anti-mouse IgG

(Amersham Corp.) for 30 mins at room temperature. Substrate was added using the ECL reagent kit (Amersham Corp) and exposed to X-ray film for 1-5minutes.

*Immunocytochemistry.* For immunohistochemical analysis of protein expression tissues were analyzed using modifications of established protocols (Ausubel 1995). Briefly, tissues were removed, fixed in 10% formalin for  $\geq 24$  hrs and desiccated. Following desiccation, tissue samples were embedded in liquid paraffin and cooled. Paraffin-embedded tissue was cut into 6  $\mu$ M sections and placed on silanized slides (Fisher Scientific). After deparaffinizing, the sections were rehydrated through increasing concentrations of xylene and alcohol and microwaved for 15 mins in 100 mM citrate buffer (pH 6.0). To reduce background signals, the slides were treated with 1% normal serum for 15 mins. After this blocking step, slides were incubated a 1:100 dilution of a rabbit anti human RET-antibody (Santa Cruz Biologicals) or 1:500 dilution of mouse monoclonal anti-human thyroglobulin antibody (Harlan Sera-Lab) overnight at room temperature. The following day, all slides were washed two times for 5 minutes each with phosphate buffered saline (PBS) and once for 5 min with PBS/1% bovine serum. Samples were incubated with biotinylated secondary antibody for one hour at room temperature, washed and incubated with substrate according to the DAB Vectastain kit (Vector Labs, Inc.), counterstained using hematoxylin, dehydrated and mounted.

## Results

### Thyroid-specific expression of the RET/PTC3 mRNA and protein in transgenic mice

Three founder lines were produced (designated 3214, 3218 and 3209) using a bovine thyroglobulin promoter construct (Figure 1) engineered to provide organ specific expression in murine thyroids. Each founder line was backcrossed to C57BL/6 mice and F<sub>1</sub> progeny typed using PCR with transgene-specific primers. RPA was performed to determine the expression of *RET/PTC3* mRNA in transgenic thyroids because the low level of gene expression provided by the bovine thyroglobulin (Jhiang, Sagartz et al. 1996; Portella, Salvatore et al. 1996; Rochefort, Caillou et al. 1996; Santoro, Chiappetta et al. 1996) and the false positive results associated with RT-PCR of cDNA. RPA results show thyroid-specific expression of the transgene is specific for the thyroid (Figure 2). Each line showed similar levels of transgene expression when normalized to b-actin controls (Figure 2). Western blot analysis using an anti-human RET antibody confirmed full-length RET/PTC3 protein expression in thyroids of transgenic mice (Figure 3A). In addition, phosphorylated RET/PTC3 protein was detected using an anti-phosphotyrosine antibody (Figure 3), consistent with previous reports of RET/PTC autophosphorylation (Pandey, Liu et al. 1996). The phosphoprotein analysis indicated that an unknown phosphoprotein (also present in nontransgenic mice) comigrates with RET/PTC3 and contributes to approximately 30 percent of the signal (Figure 3B comparison of lanes 4 and 5). This unknown protein is not the endogenous  $M_r$  140,000 or  $M_r$  160,000 murine Ret protein (Pandey, Liu et al. 1996) and thus these data support the finding that the observed  $M_r$  71,000 RET/PTC3 protein is phosphorylated in transgenic mice.

### Thyroid follicular cell hyperplasia in RET/PTC3 transgenics

Three independent transgenic lines expressing the *RET/PTC3* transgene (Figure 1) were identified and examined for pathological changes in the thyroid. Starting at 2 months of age, gross analysis of thyroids revealed frequent hypertrophy of the thyroid gland with each lobe averaging three to four times normal size (Figure 4). This observed glandular hypertrophy was due to follicular cell hyperplasia in 69 percent of transgenics <3 months of age (Table 1). Microscopic analysis of thyroid tissue showed hyperplasia of the follicles as evidenced by large colloid-containing regions and an increase in follicular cells compared to normal thyroid controls (Figure 5). Although the architecture of transgenic thyroids was severely altered, thyroglobulin production was active and evident as demonstrated by anti-thyroglobulin positive staining in the follicles (Figure 5D). Although distinguishing malignant from non-malignant tissue in murine thyroids can be difficult, thyroids from young mice (3 months of age) showed signs of cellular transformation characterized as follicular cell hyperplasia and dysplastic follicles. These data demonstrate that the expression of *RET/PTC3* caused widespread follicular cell hyperplasia leading to architectural abnormalities in transgenic thyroids.

### Solid Subtype of Papillary Thyroid Carcinoma

Mice older than 3 months of age were sacrificed and thyroids examined for pathological changes. A majority of animals examined showed evidence of secondary hyperplasia (cellular invaginations within follicles) and carcinoma (Table 1). Papillary thyroid carcinomas in transgenic mice were evident as cellular nodules adjacent to, or

extending into, colloid filled follicles (Figures 6A and B). Thyroid carcinomas in *RET/PTC3* transgenic mice were unusual because they presented as large regions of tissue devoid of follicles or papillae (Figure 6C). Immunocytochemical analysis of primary tumors revealed RET-positive follicular cells (Figure 6B) as well as RET-positive regions of solid tumor tissue with few or no papillae (Figure 6C). Cytoplasmic expression of RET/PTC3 in *RET/PTC3* transgenic follicular cells was observed following staining with the RET-specific antibody but absent in neither control antibody-stained serial sections nor normal thyroid control tissue (Figure 7 and not shown). Furthermore, the solid regions of tumor that contained homogenous populations of tumor cells also expressed high levels of RET/PTC3 protein, as evidenced by specific anti-RET antibody staining (Figure 7).

#### Metastatic Papillary Thyroid Carcinoma

In randomly selected mice, lymph nodes were examined for signs of metastasis. An example of a large metastatic tumor, identified as a 1 cm<sup>3</sup> derived from an axillary lymph node of a 10 month old transgenic, is shown in Figure 8. This metastatic tumor showed papillary features reminiscent of human papillary thyroid carcinoma (Figure 8A) as well as regions containing homogeneous populations of tumor cells (Figure 8B) similar to primary thyroid tumors from transgenic mice (Figures 8C and D). Immunohistochemical examination using antibodies specific for murine thyroglobulin showed positive staining supporting a differentiated phenotype of the metastatic tumor tissue (Figure 8A and B). Northern blot analysis of metastatic tumor RNA indicated high levels of the *RET/PTC3* transgene expression (Figure 9). This tumor also stained positive

for RET protein (not shown). In all 33 percent (2/6) mice examined showed signs of metastasis (Table 1). These data show that, in addition to causing an early onset thyroid carcinoma followed by locally invasive carcinoma, RET/PTC3 protein-expressing tumors can metastasize to regional lymph nodes. These data are consistent with the high frequency of lymph node metastasis in the morphologically related solid-type insular carcinomas in humans (Nikiforov and Gnepp 1994).

## Discussion

Establishing murine models of human disease is essential for the development of therapies and for the enhancement of disease diagnosis. In this study we were interested in developing a mammalian model of *RET/PTC3* function. The thyroid-specific expression of the active *RET/PTC3* gene and protein was confirmed and pathological sequelae were noted. All transgenic lines developed follicular cell hyperplasia early in life, which in a majority of cases progressed to invasive carcinoma. Primary tumors were characterized by small papillary structures with most of the tumor tissue growing into a sheet of solid cells. Metastatic tissue invaded regional lymph nodes and retained differentiated features of thyroid follicular cells. This was strikingly similar to the follicular abnormalities and papillary carcinomas characteristic of those observed in children (Zimmerman 1997).

Even though papillary thyroid carcinoma has been divided into different histological subtypes, little is known about the genes responsible for these morphological differences. The analysis of thyroid tumors described in Belarussian children exposed to high levels of radiation (Zimmerman 1997) suggested that these tumors were more invasive with histopathological features distinct from sporadic papillary carcinoma and consistent with the thyroid histology observed in children from other geographical regions (Harach and Williams 1995). These tumors were characterized by their solid tumor appearance (Nikiforov and Gnepp 1994), were locally invasive and demonstrated a high frequency of cervical metastasis (Ito, Yamashita et al. 1996). Most notably, the tumors identified in the Belarussian children who were exposed to ionizing radiation expressed the *RET/PTC3* oncogene in a majority (58%) of the papillary carcinomas,

which comprised ~40% of the total thyroid malignancies observed (Nikiforov, Rowland et al. 1997). Interestingly, we also observed a high frequency of solid/insular type papillary carcinomas in the thyroids of *RET/PTC3* transgenic mice that developed malignancy. In fact, all mice that developed carcinoma showed solid tumor appearances in 25-75% of the tumor mass (data not shown). This is significant in light of the similarity with papillary cancer in *RET/PTC3* expressing human tumors (Nikiforov and Gnepp 1994; Fugazzola, Pilotti et al. 1995; Harach and Williams 1995; Klugbauer, Lengfelder et al. 1995; Ito, Yamashita et al. 1996; Klugbauer, Lengfelder et al. 1996; Nikiforov, Rowland et al. 1997; Zimmerman 1997) and the fact that no previous transgenic model has demonstrated metastatic papillary cancer using a single human oncogene. In addition, *RET/PTC1* transgenic mice do not develop thyroid tumors with the histological features described herein (Jhiang, Sagartz et al. 1996; Portella, Salvatore et al. 1996; Santoro, Chiappetta et al. 1996), suggesting that these results are specific for *RET/PTC3*. Although metastatic carcinomas have been observed in a papillomavirus E1A/A2a adenosine receptor double transgenic mouse strain, these genes have not been directly implicated in any form of human thyroid cancer (Coppee, Gerard et al. 1996). These data suggest that the *RET/PTC3* protein may influence the behavior of thyroid carcinoma cells causing the development of invasive metastatic tumors similar in morphology to radiation-induced thyroid cancers observed in children and sporadic thyroid cancers in adults.

The studies reported herein show that the cellular changes caused by the expression of *RET/PTC3* protein in the murine thyroid leads to thyroid carcinoma with differentiated features (e.g. thyroglobulin expression). The hypothesis that the *RET/PTC3*

gene is critical to the development of the solid subtype of thyroid carcinoma is supported by the observation that thyroid carcinomas in children expressing *RET/PTC3* showed a high percentage of solid tumor appearances, including high rates of metastatic spread (Hassoun, Hay et al. 1997). The fact that these tumors are reportedly more invasive than the more indolent differentiated papillary carcinomas suggests that the *RET/PTC3* fusion protein confers this increased malignant phenotype in transformed thyroid follicular cells.

The *RET/PTC3* transgenic mice provide an ideal model system to study the genes that distinguish the variants of papillary thyroid carcinoma as well as the pathways controlling follicular cell differentiation. Indeed, the continued study of these transgenic mice will allow a detailed analysis of the events that lead to the initiation and progression of papillary thyroid carcinoma and the cause of malignancy, tumor progression and phenotypic variation observed in human papillary thyroid tumors.

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## Figure Legends

Figure 1. Schematic of the construct used in the generation of thyroid-specific *RET/PTC3* transgenic mice. TG-promoter, thyroglobulin promoter region; *RFG*, Ret-fused gene or *ele1*; *c-Ret*, the kinase region of the *c-RET* proto-oncogene located in the carboxy terminus of the gene; pA, SV40 poly adenylation signal sequence. The construct shown was ligated into the pBluescript II-SK<sup>+</sup> vector<sup>TM</sup> (Stratagene, Inc.) for propagation in bacterial cells.

Figure 2. RNase protection analysis of *RET/PTC3* transcripts. RNA was extracted from transgenic and nontransgenic thyroids as described in Materials and Methods. Transgene-specific (lanes 6-10) or  $\alpha$ -actin specific (lanes 1-5) riboprobes were hybridized to total thyroid RNA derived from transgenic line #3209, lane 1 and 6; #3214, lane 2 and 7; line #3218, lane 3 and 8; nontransgenic, lane 4 and 9; or total RNA from nontransgenic mouse liver, lane 5 and 10. Products were resolved on a 5% polyacrylamide, dried and exposed to X-ray film. The sizes of the protected fragments were 525 bp for *RET/PTC3* and 330 bp for  $\beta$ -actin.

Figure 3. Western blot analysis of RET/PTC3 protein and phosphoprotein expression in transgenic thyroids. Western blot analyses were performed with anti-RET antibody (left) or with anti-phosphotyrosine antibody (right) on thyroid cell lysates from *RET/PTC3* transgenic (lanes 2,3,5, and 6) or B6C3F1 control mice (lanes 1 and 4). Expression of the 71 KD RET/PTC3 fusion protein was major band shown in anti-RET treated samples. A

71 KD phosphoprotein was also detected in transgenic thyroids representing the autophosphorylation of the activated Ret tyrosine kinase (27, 37, 38).

Figure 4. Enlarged thyroid from transgenic mice. Mice transgenic for the *RET/PTC3* gene develop greatly enlarged thyroid glands between 2-8 months of age. Shown is a representative thyroid from a transgenic animal (left) compared to a normal thyroid (right).

Figure 5. Thyroid follicular abnormalities in transgenic mice. Formalin-fixed-paraffin-embedded murine thyroids were either stained with hematoxylin & eosin or with a thyroglobulin-specific antibody to identify colloid containing thyroid follicles. A) Normal mouse thyroid; B) Same normal thyroid specimen as in A stained with thyroglobulin antibody. C) Representative transgenic thyroid showing characteristic follicular cell hyperplasia defined as numerous small follicles composed of proliferating epithelial cells with associated neoplastic nodules. D) Same as C stained with anti-thyroglobulin antibody. Magnification shown is 10X.

Figure 6. Secondary hyperplasia and solid-type carcinoma in transgenic thyroids. A) Thyroid from a *RET/PTC3* transgenic shows evidence of secondary hyperplasia and carcinoma (cluster of darkly stained cells on the right side of figure). B) Transgenic thyroid demonstrating secondary hyperplasia and carcinoma growth within follicles highlighted by anti-Ret antibody staining (expression of *RET/PTC3* protein). C) A

representative transgenic thyroid showing a larger outgrowth of carcinoma from a region containing RET-positive hyperplastic thyroid follicles. Magnification shown is 10X.

Figure 7. Ret specific protein analysis of the transgenic thyroid tissue seen in Figure 6C. Hyperplastic thyroid follicles are stained with A) antibody control or with B) anti-Ret specific antibody in consecutive serial sections. Note fusion protein expression detected by anti-RET antibody staining in the cytoplasm of follicular cells. A representative thyroid carcinoma resected from a transgenic mouse revealing a solid tumor appearance stained with C) antibody control or with D) anti-Ret antibody indicating the high level of gene expression in these tumors. Antibody controls represent secondary antibody staining alone (no primary antibody). Magnification shown is 40X.

Figure 8. Solid-type carcinoma morphology in primary transgenic thyroid tumors and metastatic lesions. A) An axillary metastatic tumor derived from a transgenic mouse stained with anti-thyroglobulin specific antibody (10X). B) A higher power view of the specimen shown in A stained with anti-thyroglobulin antibody (40X). The solid appearance of these tumors can be seen in the upper right corner of the specimen in A and at high power in B. Anti-thyroglobulin antibody negative controls not shown. C) Hematoxylin and eosin stained thyroid carcinoma specimen from a representative transgenic mouse depicting the undifferentiated solid tumor appearance of an advanced papillary thyroid carcinoma. D) Higher power view of the same specimen shown in C (40X).

Figure 9. Northern blot analysis of *RET/PTC3* expression in metastatic thyroid carcinoma in axillary lymph nodes. RNA from normal mouse thyroid, lane 1; normal mouse salivary gland, lane 2; and axillary tumor, lane 3, was isolated and 10  $\mu$ g resolved on a 1.2 % formaldehyde agarose gel, blotted onto nylon membrane and hybridized with a *RET/PTC3* cDNA probe (upper) or with a mouse  $\beta$ -actin probe as control (lower). The autoradiographic result after 24 hr exposure is shown.

Tables and Figures

**Table 1.** *Pathological Abnormalities observed in RET/PTC3 thyroids*

| Thyroid pathology                   | Number of mice with pathological abnormalities/total examined at age (%) |            |
|-------------------------------------|--|------------|
|                                     | <3 months  | >3 months  |
| Follicular hyperplasia <sup>a</sup> | 9/13 (69%)   | 5/11 (45%) |
| Papillary carcinoma <sup>b</sup>    | 4/13 (31%)   | 6/11 (55%) |
| Metastatic carcinoma <sup>c</sup>   | 0/6 (0%)   | 2/6 (33%)  |

<sup>a</sup> Follicular cell hyperplasia was defined as increased numbers of thyroid follicular cells and large colloid-containing follicles. In addition, secondary hyperplasia was observed in these specimens defined as papillary invaginations of cells within follicles.

<sup>b</sup> Papillary carcinoma was characterized as disorganized cellular nodules (greater than 50-100 cells) bordering or extending into the thyroid follicle with some or no papillary structures, and/or large regions of tumor cells (5-10% or more of thyroid size) devoid of follicular or papillary structures.

<sup>c</sup> Metastatic carcinoma was determined based on cellular morphology, anti-Ret, and anti-thyroglobulin staining in dissected axillary lymph nodes.



Figure 1



Figure 2

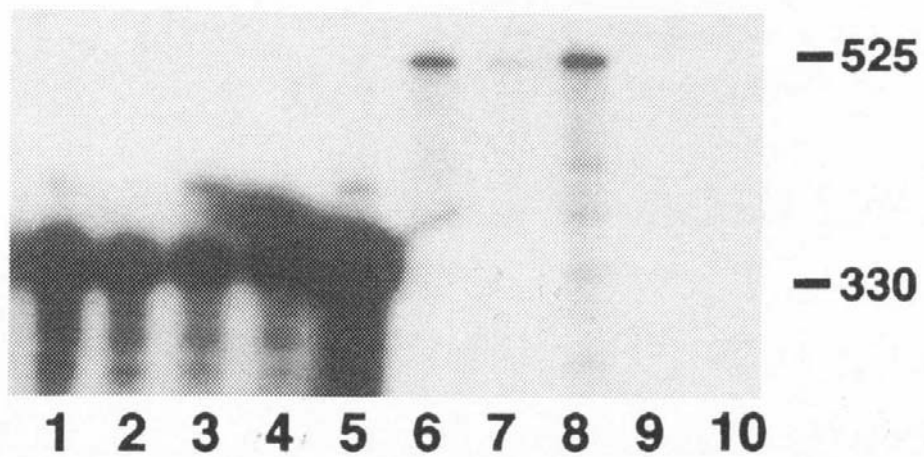


Figure 3

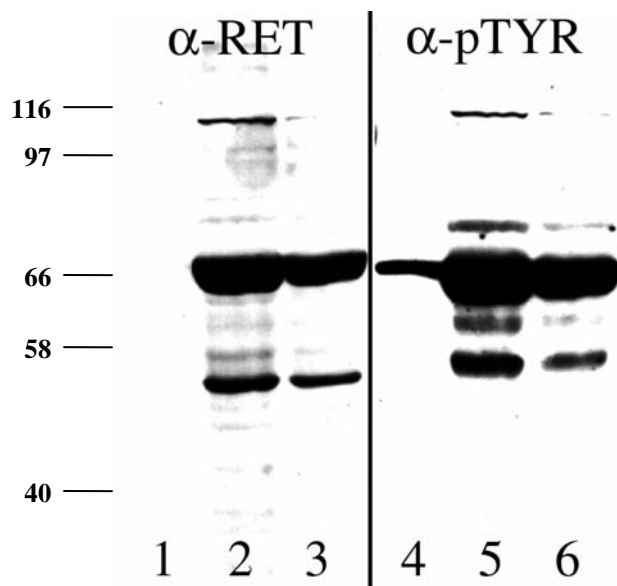


Figure 4



Figure 5

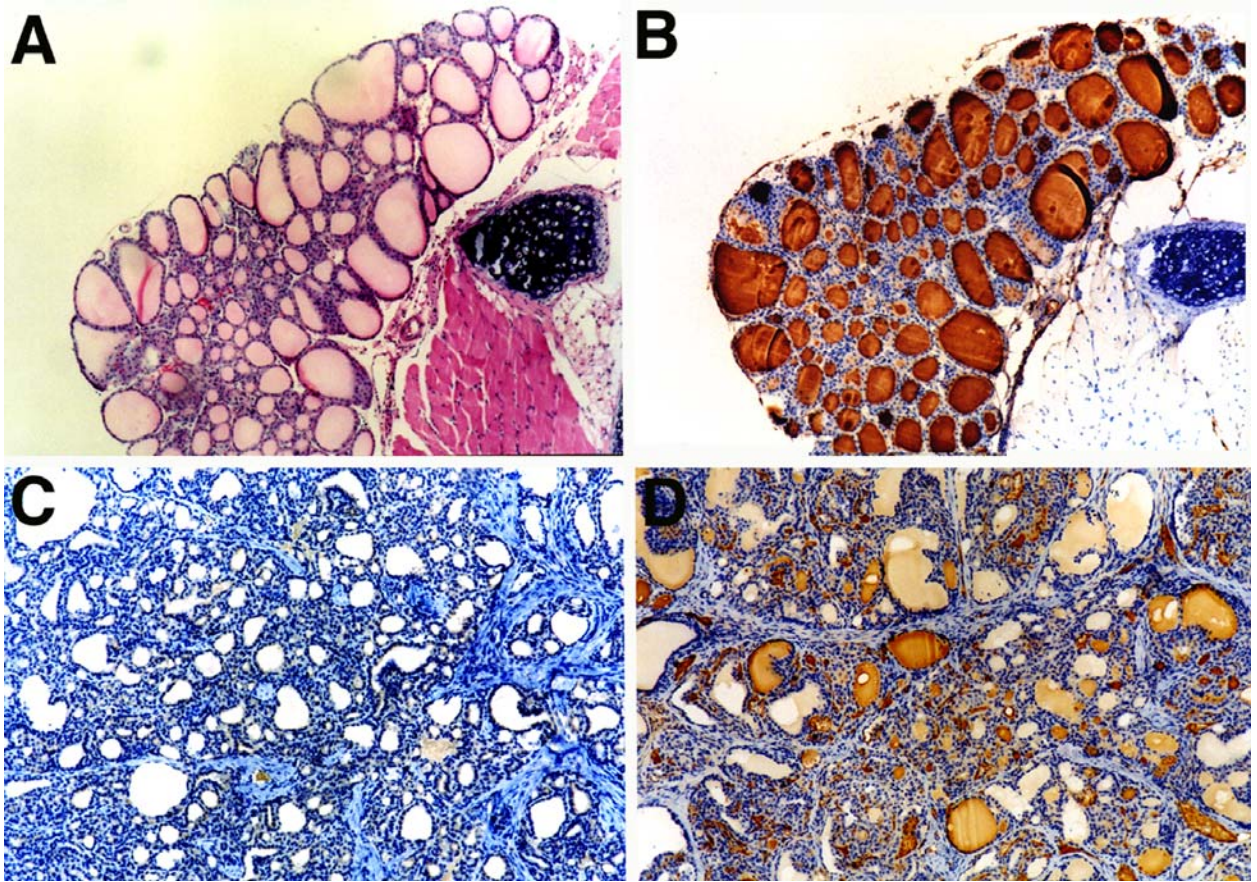


Figure 6

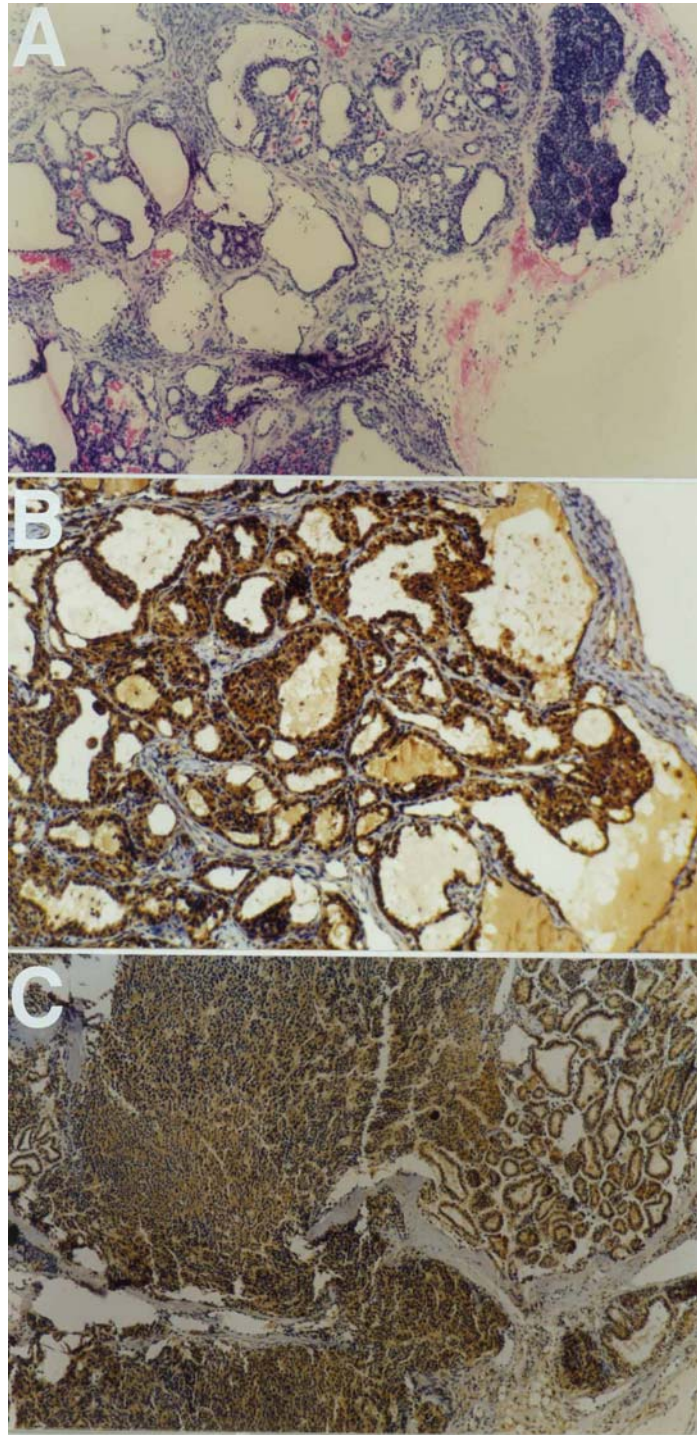


Figure 7

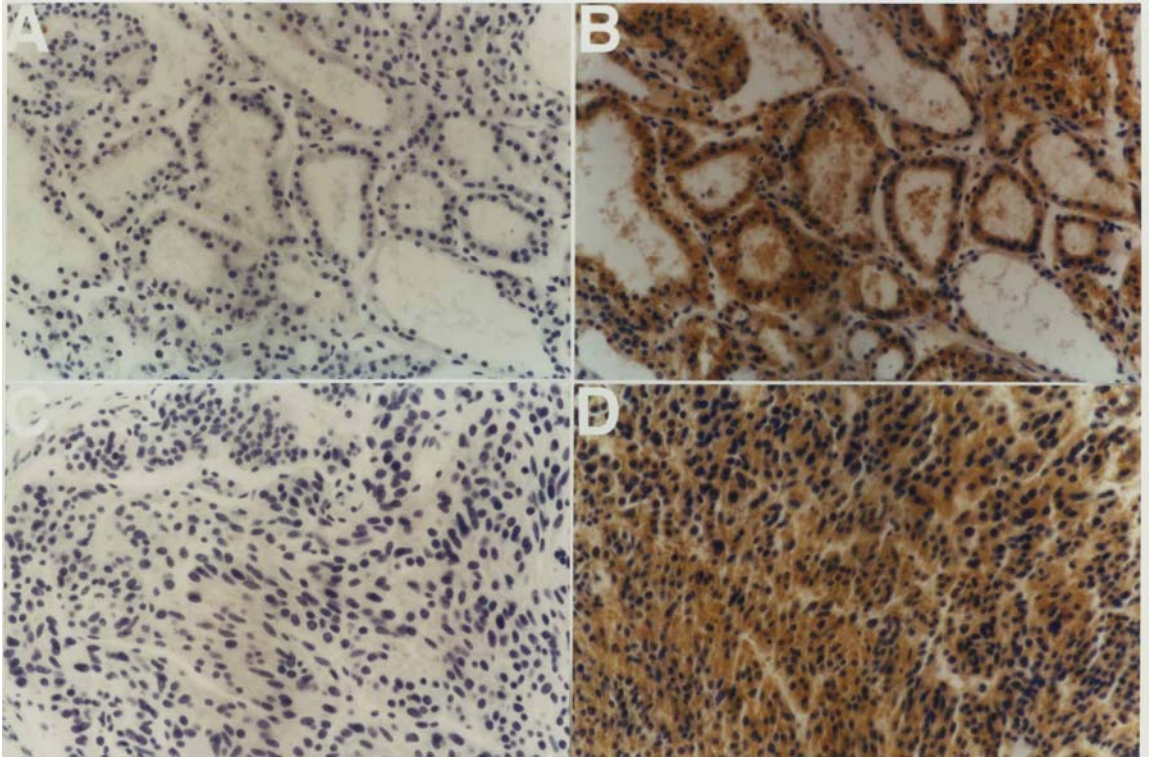


Figure 8

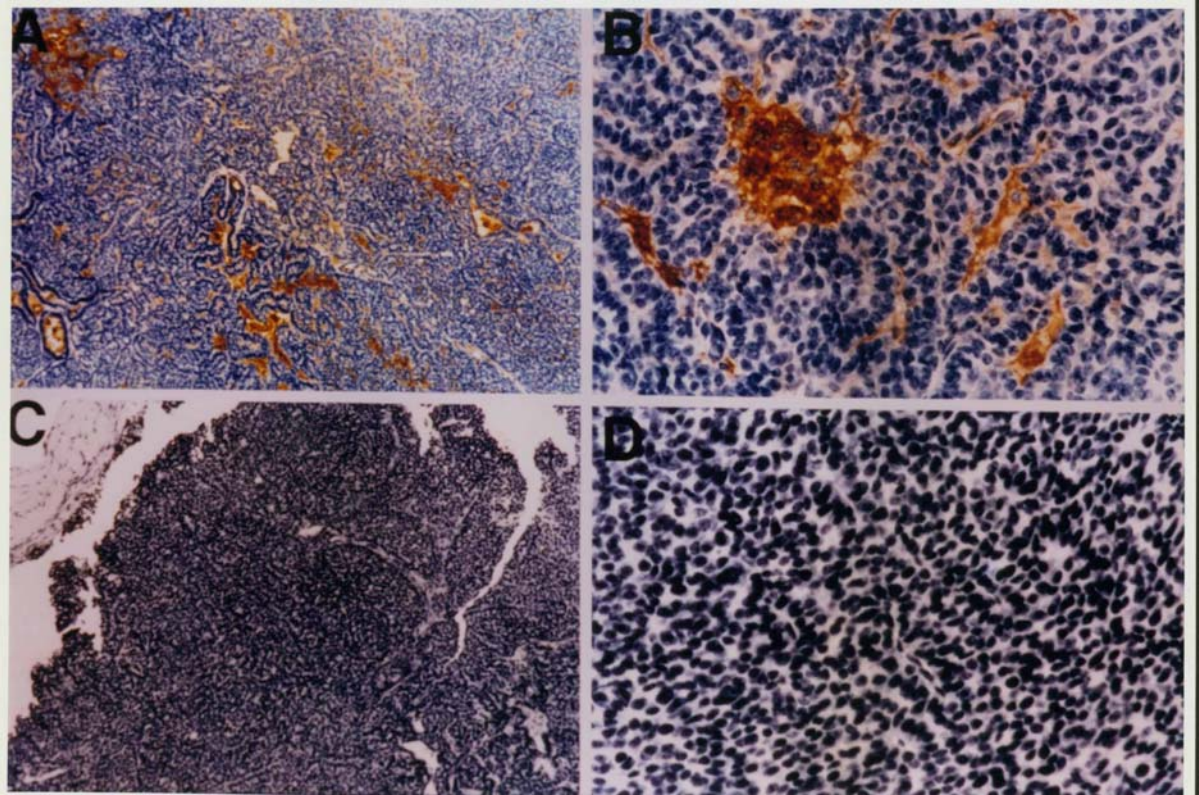


Figure 9

