The \textit{RET/PTC3} Oncogene: Metastatic Solid-Type Papillary Carcinomas in Murine Thyroids\textsuperscript{1}

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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 five members of this family have the \textit{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 \textit{RET/PTC} fusion protein action, we have devised a molecular genetic strategy to study PTC using a mouse model of thyroid disease. A new member of this fusion oncogene family, \textit{RET/PTC3}, which has been implicated in more cases of solid tumor carcinoma (79\%) than \textit{PTC1} or \textit{PTC2} and predominates (80\%) in radiation-induced thyroid cancer of children, was investigated in our study. We have generated transgenic mice expressing human \textit{RET/PTC3} exclusively in the thyroid. These mice develop thyroid hyperplasia, solid tumor variants of papillary carcinoma and metastatic cancer. 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 United States with the papillary subtype accounting for at least 80\% of all thyroid cancers (1). Chromosomal translocations were identified in these tumors, and breakpoints were cloned and sequenced. The common characteristic of these gene rearrangements is the expression of fusion proteins, which contain the COOH-terminal kinase domain of either the \textit{c-RET} or TRK gene families (2-4). The invariant expression of the kinase domain in the \textit{RET-type} fusion proteins, collectively called \textit{RET/PTC}, suggests an important role for these genes in the transformation of thyroid follicular cells (4). Several studies have implicated the role of \textit{RET/PTC} fusion genes early in thyroid carcinogenesis (2, 4-18), and multiple independent rearrangements have been detected in the same tumor specimen, suggesting that these gene products have implicated the role of \textit{RET/PTC} fusion genes early in thyroid carcinogenesis (2, 4-18). Furthermore, \textit{RET/PTC1} transgenic mice develop follicular hyperplasia and carcinoma, although invasive cancer was not observed (13, 21, 22).

The most recent analysis of children exposed to radiation after the Chernobyl disaster has indicated a high number of thyroid carcinomas expressing the \textit{RET/PTC3} fusion gene (11, 14, 15). 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 (19). Thus, to investigate the role of \textit{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 \textit{RET/PTC3} gene under the control of the bovine thyroglobulin promoter (20). These transgenic mice develop thyroid hyperplasia and solid tumor variants of papillary carcinoma with metastatic spread in selected cases. Transgenic mice express the \textit{RET/PTC3} fusion protein in thyroid follicular cells as determined by immunohistochemical analysis. These new \textit{RET/PTC3} mice will be a useful resource for the study of thyroid cancer and the behavior of the different histological variants.

MATERIALS AND METHODS

Generation of Transgenic Mice. Constructs for the generation of transgenic mice were cloned into the pBluescript-II SK\textsuperscript{+} vector. Plasmid DNA was purified using Qiagen columns according to the manufacturer’s protocol (Qiagen, Inc., Santa Clarita, CA). A DNA construct was made that contained the bovine thyroglobulin promoter (20), the \textit{RET/PTC3} coding sequence, and an SV40 polyadenylation signal (Fig. 1). For transgenesis, 2 \mu g of purified construct DNA were microinjected into zygotes as described (23). Founder animals were developed by the Kimmel Cancer Institute Transgenic Facility and identified by Southern hybridization using a \textit{RET/PTC3}-specific probe (24). Founder mice were placed into mating with wild-type C57BL/6, and progeny were examined for the presence of the transgene by PCR.

DNA Amplification by PCR. The PCR was performed by adding 5 \mu l of cDNA or 100 ng of genomic DNA to a 25-\mu l reaction mixture containing 1 \times PCR buffer, 0.2 mM deoxynucleotide triphosphates, 50 pm 3’ and 5’ oligonucleotides (5’ primer sequence of TGPRO, GCCGACAGGCTCTAAGGTTGGGC; and 3’ primer RET, GATACAGCCTCTCTTCTTCCA) and 2.0 units of Taq polymerase (24). The specimens were then placed into a heated lid thermocycler (Hybaid, Inc.) and subjected to 30 cycles of denaturation at 94\°C for 30 s, annealing at 60\°C for 30 s, and elongation at 72\°C for 60 s.

RTP.\textsuperscript{5} Thyroid tissue was removed from transgenic mice and homogenized in 0.5 ml of cell lysis buffer (4 mM guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium N-lauroylsarcosine, and 0.1 M 2-mercaptoethanol). Nucleic acid was extracted using phenol:chloroform (1:1) and ethanol was precipitated as described (24). When necessary, DNA was removed by DNase treatment (25). \textit{RET/PTC3} riboprobes were synthesized from a 525-bp cloned fragment of the \textit{RET} gene, whereas 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\% denatured formamide, 100 mM sodium citrate, pH 6.4), 300 mM sodium acetate (pH 6.4), and 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, and 0.025% SDS), denatured for 3 min at 94\°C, and run on a 5% denaturing polyacrylamide gel for 1 h at 250 V. Gels were exposed to X-ray film for 1–5 min.

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\textsuperscript{5} The abbreviation used is: RPA, RNase protection analysis.

5523

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Western Blot Analysis. Tissues were homogenized, and cells were lysed in protein extract buffer [30 mm Tris-HCl (pH 8.0), 10 mm EDTA, 1% Triton X-100, 100 mm NaCl, 1 mm phenylmethylsulfonyl fluoride] and stored at −70°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were then blocked for 1 h in TBS-T buffer (20 mm Tris-HCl [pH 7.6], 135 mm NaCl, and 1% Tween 20) and incubated at 4°C overnight with anti-RET antibody preabsorbed with protein extract from whole nontransgenic thyroids (diluted 1:1,000) or anti-phosphotyrosine (diluted 1:1,000) antibodies (Santa Cruz Biotechnology). After incubation, the membrane was treated with either homenadish peroxidase-conjugated anti-rabbit IgG (Promega) or anti-mouse IgG (Amersham Corp.) for 30 min at room temperature. Substrate was added from the ECL reagent kit (Amersham Corp.) and exposed to X-ray film for 1–5 min.

Immunocytochemistry. For immunohistochemical analysis of protein expression, tissues were analyzed using modification of established protocols (24). Briefly, tissues were desiccated and fixed in 10% formalin for 24 h. After fixation, tissue samples were embedded in liquid paraffin and cooled. Paraffin-embedded tissue was cut into 6 μm sections and placed on silanized slides (Fisher Scientific). After deparaffinization, the sections were rehydrated through increasing concentrations of xylene and alcohol and microwaved for 15 min in 100 mm citrate buffer (pH 6.0). To reduce background signals, the slides were treated with 1% normal serum for 15 min. After this blocking step, slides were incubated with a 1:100 dilution of a rabbit anti-human RET antibody (Santa Cruz Biologicals), specific for an epitope corresponding to amino acids 784–801 mapping at the COOH terminus of the RET/PTC protein (26), or 1:500 dilution of mouse monoclonal anti-human thyroglobulin antibody (Harlan Sera-Lab, Loughborough, UK) overnight at room temperature. The following day, all slides were washed twice for 5 min each with PBS and once for 5 min with PBS/1% bovine serum. Samples were incubated with biotinylated secondary antibody for 1 h 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 (Fig. 1) engineered to provide organ-specific expression in murine thyroids. Each founder line was backcrossed to C57BL/6 mice and F1 progeny typed using PCR with transgene-specific primers. RPA was performed to determine the expression of RET/PTC3 mRNA in transgenic thyroids because of the low level of gene expression provided by the bovine thyroglobulin promoter (13, 20–22) and the false-positive results associated with RT-PCR of cDNA. RPA results show thyroid-specific expression of the transgene (Fig. 2). Each line showed similar levels of transgene expression when normalized to β-actin controls (Fig. 2). Western blot analysis confirmed full-length RET/PTC3 protein expression in thyroids of transgenic mice (Fig. 3). In addition, phosphorylated RET/PTC3 protein was detected using an anti-phosphotyrosine antibody (Fig. 3), consistent with previous studies of RET/PTC autophosphorylation (27). The phosphoprotein analysis indicated that an unknown phosphoprotein (also present in nontransgenic mice) comigrates with RET/PTC3 and contributes to ~30% of the signal (Fig. 3, right panel, compare Lanes 4 and 5). This unknown protein is not the endogenous M, 140,000 or M, 160,000 murine Ret protein (28), and thus these data support the finding that the observed M, 71,000 RET/PTC3 protein is phosphorylated in transgenic mice.

Thyroid Follicular Cell Hyperplasia in RET/PTC3 Transgenics. All three transgenic lines expressing the RET/PTC3 transgene were 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 (Fig. 4). This observed glandular hypertrophy was due to follicular cell hyperplasia in 69% 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 with normal thyroid controls (Fig. 5). Although the architecture of transgenic thyroids was severely altered, thyroglobulin production was active and evident as demonstrated by antithyroglobulin-positive staining in the follicles (Fig. 5D). Although distinguishing malignant from nonmalignant 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 (Fig. 6, A and B). These data demonstrate that the expression of the RET/PTC3 transgene caused widespread follicular cell hyperplasia leading to morphological abnormalities in transgenic thyroids.

Solid Subtype of Papillary Thyroid Carcinoma. Mice 3 months of age and older were sacrificed, and thyroids were 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 (Fig. 6, A and B). Thyroid carcinomas in RET/PTC3 transgenic mice were unusual because they presented as large regions of tissue devoid of follicles or papillae (Fig.
Fig. 4. Enlarged thyroid from transgenic mice. Mice transgenic for the RET/PTC1 gene develop greatly enlarged thyroid glands between 2 and 8 months of age. Shown is a representative thyroid from a transgenic animal (left) compared with a normal thyroid (right).

Immunocytochemical analysis of primary tumors revealed RET/PTC3-positive follicular cells (Fig. 6B) as well as RET/PTC3-positive regions of solid tumor tissue with few or no papillae (Fig. 6C, left side). RET-specific staining was apparent in the cytoplasm of follicular cells (Fig. 7B) but absent in control sections (Fig. 7, A and C). Furthermore, the solid regions of the tumor that contained homogeneous populations of tumor cells also expressed high levels of RET/PTC3 protein, as evidenced by specific anti-Ret antibody staining (Fig. 7D).

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-cm³ mass derived from an axillary lymph node of a 10-month-old transgenic, is shown in Fig. 8. This metastatic tumor showed papillary features reminiscent of human papillary thyroid carcinoma (Fig. 8A) as well as regions containing homogeneous populations of tumor cells (Fig. 8B) similar to primary thyroid tumors from transgenic mice (Fig. 8, C and D).

Table 1 Pathological abnormalities observed in RET/PTC3 thyroids

<table>
<thead>
<tr>
<th>Thyroid pathology</th>
<th>No. of mice with pathological abnormalities/total examined at age (%)</th>
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<tr>
<td></td>
<td>&lt;3 mo</td>
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<tr>
<td>Follicular hyperplasia a</td>
<td>9/13 (69%)</td>
</tr>
<tr>
<td>Papillary carcinoma b</td>
<td>4/13 (31%)</td>
</tr>
<tr>
<td>Metastatic carcinoma c</td>
<td>0/6 (0%)</td>
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a Follicular cell hyperplasia was defined as increased numbers of thyroid follicular cells surrounding large colloid-containing follicles. In addition, secondary hyperplasia was observed in these specimens defined as papillary invaginations within follicles.

b Papillary carcinoma was characterized as disorganized cellular nodules (>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.

c Metastatic carcinoma was determined based on cellular morphology, RET/PTC3 expression, and antithyroglobulin staining in dissected axillary lymph nodes.

Fig. 5. Thyroid follicular abnormalities in transgenic mice. Formalin-fixed, paraffin-embedded murine thyroids were either stained with H&E or with a thyroglobulin-specific antibody to identify colloid containing thyroid follicles. A, normal mouse thyroid; B, serial section from the 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 antithyroglobulin antibody. ×10.
with the high frequency of lymph node metastasis in the morphologically related solid-subtype papillary carcinomas in humans (19).

**DISCUSSION**

Establishing murine models of human disease is essential for the development of therapies and 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. Although specific pathological features of the murine thyroid tumors may be distinguishable from human thyroid cancer, the similarities were remarkable. All transgenic lines developed follicular cell hyperplasia early in life, which in a majority of cases progressed into invasive carcinoma. Primary tumors were characterized by small papillary structures, with most of the tumor tissue growing into a sheet of solid cells. Metastatic thyroid tissue that invaded lymph nodes retained the differentiated features of thyroid follicular cells. This was strikingly similar to the follicular abnormalities and papillary carcinomas characteristic of those observed in children (29).

Although papillary thyroid carcinoma has been divided into different histological subtypes, little is known about the genes responsible for these morphological differences. The recent analysis of thyroid tumors described in Belarusian children exposed to high levels of radiation 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 (32). Indeed, these tumors were characterized by their solid tumor appearance (33), were locally invasive, and demonstrated a high frequency of cervical metastasis and a poor prognosis (30). Most notably, the tumors identified in the Belarusian 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 (19). Interestingly, we also observed a high frequency of solid-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 (14, 15, 19, 29, 30, 32–34) 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 tumors with the histological features described herein (13, 21, 22), suggesting that these results are specific for RET/PTC3. Although metastatic carcinomas have been observed in a papillomavirus E7/A2 adenosine receptor double transgenic mouse strain, these genes have not been directly implicated in any form of human thyroid cancer (35). 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.

These studies 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 (36). 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 mouse model to

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**Fig. 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 carcinomatous 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. ×10.

Immunohistochemical examination using antibodies specific for murine thyroglobulin showed positive staining, supporting a differentiated phenotype of this metastatic tumor (Fig. 8, A and B). Northern blot analysis of metastatic tumor RNA indicated high levels of the RET/PTC3 transgene expression (Fig. 9). This tumor also stained positive for Ret protein (not shown). In all, two of six (33%) mice examined showed evidence 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-subtype papillary carcinomas in humans (19).
Fig. 7. Ret-specific protein analysis of the transgenic thyroid tissue seen in Fig. 6C. Hyperplastic thyroid follicles are stained with antibody control (A) or with anti-Ret-specific antibody (B) 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 antibody control (C) or with anti-Ret antibody (D), indicating the high level of transgene expression in these tumors. Antibody controls represent secondary antibody staining alone (no primary antibody). ×40.

Fig. 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 antithyroglobulin-specific antibody (×10). B. a higher power view of the specimen shown in A stained with antithyroglobulin antibody (×40). 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. Antithyroglobulin antibody-negative controls are not shown. C. H&E-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 (×40).
study the genes that distinguish the variants of papillary thyroid carcinoma as well as the pathways controlling follicular cell differentiation. Moreover, 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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