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Targeted Expression of the *ret*/PTC1 Oncogene Induces Papillary Thyroid Carcinomas

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Abstract The *ret*/PTC oncogene, a rearranged form of the *ret* proto-oncogene, has been found to be restricted to human papillary thyroid carcinomas. This report shows that transgenic mice with thyroid-targeted expression of the *ret*/PTC1 oncogene developed thyroid carcinomas with considerable similarities to human papillary thyroid carcinomas, particularly in the nuclear cytologic features and the presence of local invasion. Our findings indicate that *ret*/PTC1 is not only a biomarker associated with papillary thyroid carcinomas, but is also the only proven specific genetic event leading to the development of papillary thyroid carcinoma.

Introduction

In human thyroid glands, there are two pathways of tumor formation originating from follicular cells, one directly to papillary thyroid carcinoma, and the other through follicular adenoma to follicular carcinoma (1). These two pathways show differences in etiology, oncogene involvement, and clinical behavior. In geographic areas where dietary iodine is sufficient, papillary thyroid carcinomas account for about 80% of thyroid cancers, and radiation exposure is the only known etiologic factor. Among oncogenes studied, the *ret*/PTC oncogene is specific to papillary thyroid carcinoma although only found in a minority of cases (2, 3).

The *ret*/PTC oncogene is a rearranged form of the *ret* proto-oncogene (4) which encodes a receptor tyrosine kinase whose ligand has yet to be identified (5). The *ret* proto-oncogene is expressed in a tissue and developmental stage-specific manner (6, 7), which suggests that the gene product may play a role in the differentiation and/or proliferation of specific tissues of the neurologic, endocrine and excretory systems (8). Indeed, germline mutations of the *ret* proto-oncogene have recently been reported to predispose the development of three variants of multiple endocrine neoplasia type II inherited cancer syndromes (9) and a congenital developmental defect of the intestine, Hirschsprung's disease (10). The tissues affected in these four syndromes are derived from cells of neural crest origin, where the *ret* proto-oncogene is constitutively or transiently expressed (6, 7).

In human papillary thyroid carcinoma, three forms of *ret*/PTC oncogene have been identified (11, 12). In all forms, *ret*/PTC activation is provided by fusion of the amino-terminus of different gene products to the tyrosine kinase domain of the *ret* proto-oncogene product. The genes providing the 5'-replaced sequence are ubiquitously expressed, therefore, the *ret*/PTC chimeric oncogene, driven by a foreign promoter, shows unscheduled expression of *ret* tyrosine kinase in thyroid follicular cells. Significantly, all *ret*/PTC oncogenes have been found to be restricted to papillary thyroid carcinoma (2, 3), although follicular thyroid carcinoma shares a common histogenesis from thyroid follicular cells. The unique involvement of *ret*/PTC in papillary thyroid carcinoma suggests that *ret*/PTC activation is a specific genetic lesion that leads to the development of human papillary thyroid carcinoma. Among the three *ret*/PTC oncogenes, *ret*/PTC1 is the most commonly detected form and results from the rearrangement between the previously unknown H4 gene and the *ret* proto-oncogene.

To investigate whether the *ret*/PTC oncogene indeed causes papillary thyroid carcinoma, we generated two lines of transgenic mice expressing the *ret*/PTC1 oncogene in the thyroid gland. Bilateral thyroid carcinomas with cellular features comparable to human papillary thyroid cancer have been found in all transgenic mice examined.

Materials and Methods

Transgene and Transgenic mice. To target the expression of *ret*/PTC1 to the thyroid gland, a hybrid gene (Tg-PTC1) was cloned into the plasmid pRc/CMV (Invitrogen, San Diego, CA). Tg-PTC1 was comprised of the bovine thyroglobulin gene promoter (-2036 to +7) and the coding region for the *ret*/PTC1 oncogene (Fig 1A). The 3.9 kb transgene DNA fragments were released by Kpn I and Pvu II digestion, isolated by gel electrophoresis, and further purified using ELUTIP minicolumns (Schleicher & Schuell, Keene, NH). The purified transgene was microinjected into 200 fertilized FVB/N mouse eggs, and these eggs were implanted into the oviduct of pseudopregnant recipient mice and allowed to develop to term. Tail cuttings or toe clippings were used to extract DNA for examining the transgenic status. Polymerase chain reaction (PCR) was performed to amplify a target DNA fragment of 203 bp using the following primers: TPC-4: GTC GGG GGG CAT TGT CAT CT, and KD-2: AGT TCT TCC GAG GGA ATT CC. Mice shown to be PCR-positive for the transgene were further verified by genomic Southern blot analysis using a P^{32}-labeled DNA probe derived from the 3.9 kb Tg-PTC1 transgene.

Histology and Immunohistochemistry. Tissues were prepared for histopathological examination by fixation in 10% neutral-buffered formalin and embedded in paraffin. Specimens were sectioned at 5 μm and stained with hematoxylin/eosin. For immunohistochemistry, paraffin-embedded 5 μm sections were dewaxed and incubated with 0.3% H_2O_2 in methanol for 15 minutes to inactivate endogenous peroxidase. The sections were then subjected to antigen retrieval using antigen retrieval solution (BioGenex, San Ramon, CA). Non-specific staining was blocked by preincubation with 6% normal goat serum for 30 minutes. The sections were then incubated at 4°C overnight with the affinity purified polyclonal antibody against the carboxyl terminus of *ret* (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:100. Immunostaining was performed using the VECTASTAIN ABC kit with diaminobenzidine DAB (Vector Laboratories, Burlingame, CA). The sections were then counterstained with hematoxylin. Negative control for the immunostaining was carried out by replacing the primary antibody with normal rabbit serum. The specificity of the antibody

was determined by the presence of staining in the thyroid parafollicular cells which are known to express the *ret* proto-oncogene, and the absence of staining in normal thyroid follicular cells of non-transgenic mice.

Radioimmunoassays. Serum was obtained prior to euthanasia and frozen at -80°C until analysis. Radioimmunoassay for mouse TSH, T3 and T4 were performed by Ani-Lytics Inc. (Gaithersburg, MD).

Results and Discussion

Fifty eggs developed to term, three transgenic founder mice were identified, and all were phenotypically normal. These transgenic mice differed in the relative numbers of integrated transgenes, with #1 having relatively fewer copies than #16 and #42 (Fig 1B). For initial evaluation, transgenic lines were established from founder sire #1 and founder dam #42 to examine the effect of differences in copy number on phenotypic abnormalities.

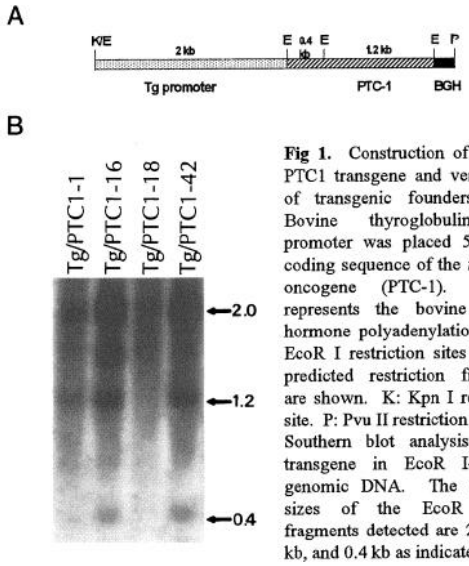

Fig 1. Construction of the Tg-PTC1 transgene and verification of transgenic founders. **(A)** Bovine thyroglobulin (Tg) promoter was placed 5' to the coding sequence of the *ret*/PTC1 oncogene (PTC-1). BGH represents the bovine growth hormone polyadenylation signal. EcoR I restriction sites (E) and predicted restriction fragments are shown. K: Kpn I restriction site. P: Pvu II restriction site. **(B)** Southern blot analysis of the transgene in EcoR I-digested genomic DNA. The expected sizes of the EcoR I-DNA fragments detected are 2 kb, 1.2 kb, and 0.4 kb as indicated.

In the low copy number line, all transgenic offspring were phenotypically normal. At approximately 7 months of age, sire #1 was found moribund and was sacrificed. Microscopic examination revealed bilateral thyroid carcinomas. Six F1 transgenic mice were sacrificed between one and six months of age. All mice developed bilateral thyroid carcinomas which were invasive into surrounding tissues and elicited a modest amount of fibrous stroma (Fig 2A). These tumors contained a mixture of solid, cribriform, and follicular architecture, with a minor degree of papillary infolding (Fig 2B). Colloid formation was poor or absent. The nuclei were variable in size, irregularly shaped, with relatively frequent "grooving" in oval nuclei, and occasional intranuclear cytoplasmic inclusions (Fig 2C). A few follicles showed cilia formation. Non-neoplastic thyroid tissues had slight nuclear irregularity in the follicular cells.

In the high copy number line (dam #42), all transgenic offspring had marked congenital hypothyroidism, as evidenced by dwarfism (Fig 3A and 3B), low serum thyroxine (T4) and triiodothyronine (T3) levels, and marked elevations in serum TSH (Fig 3C). Furthermore, the pituitary glands had marked hypertrophy and hyperplasia of thyrotrophs, indicating a lack of negative feedback response to the low thyroid hormone levels with activation of the hypothalamic-pituitary-thyroid axis. Immunohistochemical staining of the pituitary for TSH revealed that 90% of cells in the pars distalis were thyrotrophs (data not shown). Indeed, T4 supplementation was able to correct the phenotypic dwarfism (Fig 3D), and was essential for the survival and efficient breeding of transgenic mice in the high copy number line. Untreated male transgenic mice developed penile prolapse (paraphimosis) with edema and ulceration at approximately 45 days of age, and were sacrificed due to complications related to this lesion. As with the low copy number line, all transgenic mice examined from high copy number line developed bilateral thyroid carcinomas with histological features comparable to human papillary thyroid carcinomas as early as 1 month of age. Immunohistochemical staining of the thyroid glands revealed that the *ret*/PTC1 oncoprotein was present in thyroid carcinomas from both transgenic lines (Fig 2D).

The cause of congenital hypothyroidism in the high copy number line is unknown, but could be explained by two alternate mechanisms: (a) de-differentiating effect of the *ret*/PTC1 oncoproteins on the thyroid follicular cells (13), and (b) competition for trans-acting elements between the mouse endogenous thyroglobulin gene promoter and the bovine thyroglobulin gene promoter in the transgene (Tg-PTC1). Both hypotheses could explain the differences between the low copy number line (no apparent phenotypic change) and high copy number line (congenital hypothyroidism with dwarfism). Immunohistochemistry demonstrated more intense staining for *ret*/PTC1 oncoproteins in the thyroid carcinomas of the high copy number line than the low copy number line (data not shown). This phenomenon may be explained simply by higher numbers of integrated transgene or by increased expression of transgene secondary to elevated serum TSH level resulting from the severe hypothyroidism. Although both bovine and rat thyroglobulin gene promoters have been used to target thyroid-specific expression of various transgenes (14), congenital hypothyroidism has been found only in transgenic mice expressing simian virus-40 large T-antigen, an animal model that develops moderate to poorly differentiated thyroid adenocarcinomas (15). These data would argue against the hypothesis that promoter competition is the sole mechanism underlying the congenital hypothyroidism. It is more likely that the nature of transgene and its level of expression both play important roles in the development of congenital hypothyroidism.

The finding that targeted expression of *ret*/PTC1 oncogene in the thyroid glands caused bilateral thyroid carcinoma with cellular features comparable to human papillary thyroid carcinoma is significant because it indicates that *ret*/PTC1 is not only a biomarker associated with papillary thyroid cancer, but is also the only proven specific genetic event leading to the development of papillary thyroid carcinoma. Genetically, the tumors in these transgenic mice are different from human papillary

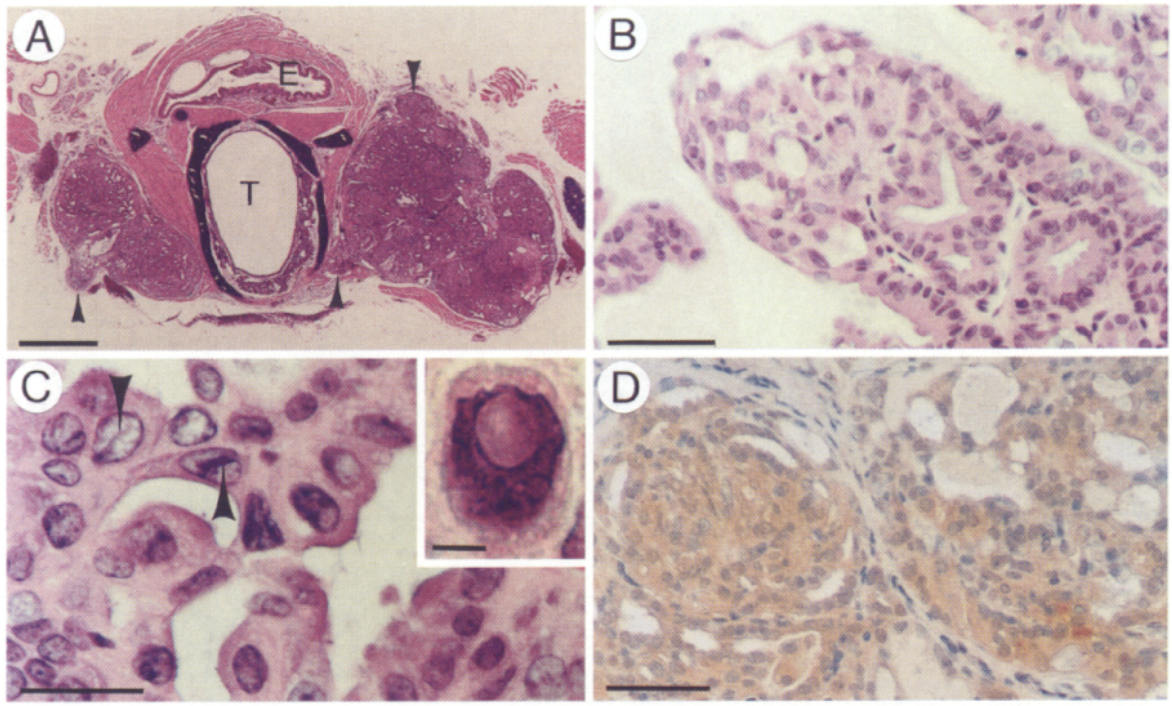

Fig 2. Histologic features of thyroid carcinomas in transgenic mice. **(A)** Bilateral thyroid carcinomas were observed in all transgenic mice from both lines. These tumors were invasive into surrounding tissue (arrows), and elicited the production of fibrous stroma. Hematoxylin-eosin (H&E) stained transverse section of the trachea (T), esophagus (E), and thyroid gland. Bar = 800 μm. **(B)** Papillary projection of thyroid carcinoma into a cystic follicle. H&E stain, bar = 60 μm. **(C)** Characteristic nuclear grooves (arrow heads), vesicular nuclei, and pseudo-inclusions (inset). H&E stain, bar = 25 μm, inset bar = 5 μm. **(D)** Immunohistochemical staining of thyroid carcinoma for the *ret*/PTC1 oncoproteins. Note diffuse cytoplasmic staining restricted to neoplastic tissue. Hematoxylin counterstain, bar = 60 μm.

thyroid carcinomas in that (a) *ret*/PTC1 is the tumor initiator in these mice, whereas in human tumors, this may not be the case; and (b) these mice may lack other specific genetic lesions that are characteristics of human papillary thyroid carcinomas. Although identity of appearance between human and animal tumors predisposed by the same oncogene would not necessarily be predicted, the thyroid tumors in these mice have considerable similarities to human papillary thyroid carcinoma, particularly in the nuclear features and local invasion. Despite a lack of evidence of local or distant metastasis in mice up to 5 months of age, intrathyroidal and periglandual invasion was present in all mice examined. The potential contribution of hypothyroidism and the role of T4 treatment, acting through the modulation of serum TSH level, in tumor promotion are currently under investigation. In summary, these transgenic mice represent the only current animal model of human papillary thyroid carcinoma and will be highly effective for investigating additional genetic lesions as well as the roles of various epigenetic events in tumor progression.

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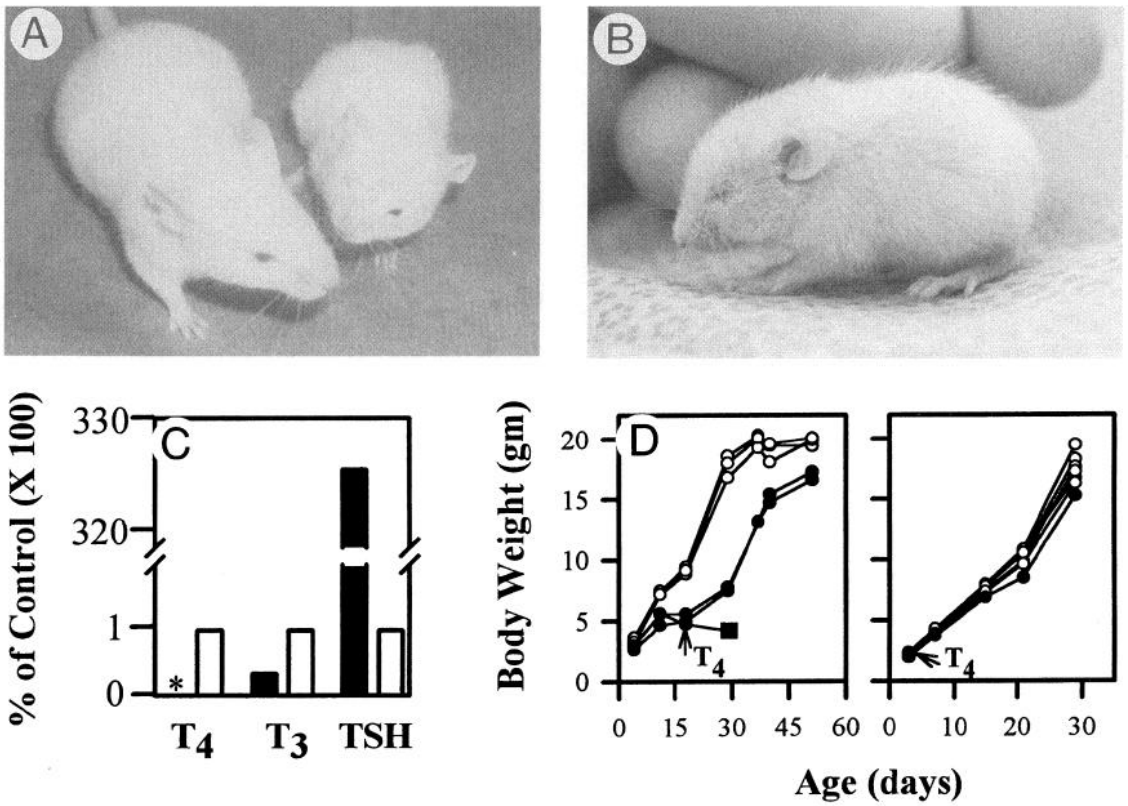

Fig 3. Congenital hypothyroidism in high copy number line of transgenic mice. **(A)** 30-day-old transgenic and non-transgenic littermates showing marked dwarfism in transgenic mouse. **(B)** Dwarfism observed in transgenic mice was accompanied by a rough hair coat, as well as facial deformities (domed forehead and short, broad nose). **(C)** Mean serum T4 and T3 levels were markedly reduced in transgenic mice (solid bars, n = 2) versus their age and sex-matched littermates (open bars, n = 4). Serum T4 (*) was from only one transgenic mouse; the serum T4 level of the other transgenic mouse was below the detection range. Serum TSH levels were markedly elevated in response to the diminished serum thyroid hormone levels. **(D)** Administration of dietary T4 (300 μg T4/kg feed) enabled near normal growth when given in the diet beginning at postnatal day 16 (left panel). When subcutaneous T4 (0.25 μg T4/g body weight/day) was administrated beginning at postnatal day 4, no dwarfism was observed (right panel). Open circles refer to non-transgenic mice, and solid circles refer to transgenic mice. The solid square indicates the time when the mouse was sacrificed.

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