Short Communication

Genetic and Biological Subgroups of Low-Stage Follicular Thyroid Cancer

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Investigations of cancer-specific gene rearrangements have increased our understanding of human neoplasia and led to the use of the rearrangements in pathological diagnosis of blood cell and connective tissue malignancies. Here, we have investigated 3p25 rearrangements of the *peroxisome proliferator-activated receptor γ* (*PPAR γ*) gene in follicular epithelial tumors of the human thyroid gland. Eleven of 42 (26%) low-stage follicular carcinomas, 0 of 40 follicular adenomas, 1 of 30 Hurthle cell carcinomas, 1 of 90 papillary carcinomas, and 0 of 10 nodular goiters had 3p25 rearrangements by interphase fluorescence *in situ* hybridization. All 11 follicular carcinomas with 3p25 rearrangement exhibited strong, diffuse nuclear immunoreactivity for *PPAR γ* , consistent with expression of *PPAR γ* fusion protein. Twelve of 42 (29%) low-stage follicular carcinomas had 3p25 aneuploidy without *PPAR γ* rearrangement ($P = 0.01$), suggesting that *PPAR γ* rearrangement and aneuploidy are independent early events in follicular cancer. Eleven of 12 follicular carcinomas with 3p25 aneuploidy exhibited no *PPAR γ* immunoreactivity, supporting the existence of two independent pathways. Follicular carcinoma patients with *PPAR γ* rearrangement more frequently had vascular invasion ($P = 0.01$), areas of solid/nested tumor histology ($P < 0.001$), and previous nonthyroid cancers ($P < 0.01$)

compared with follicular carcinoma patients without *PPAR γ* rearrangement. Our experiments identify genetic subgroups of low-stage follicular thyroid cancer and provide evidence that follicular carcinomas with *PPAR γ* rearrangement are a distinct biological entity. The findings support a model in which separate genetic alterations initiate distinct pathways of oncogenesis in thyroid carcinoma subtypes. (*Am J Pathol* 2003, 162:1053–1060)

Chromosomal rearrangements that create abnormal gene fusions are some of the most early and specific genetic alterations identified in cancer.^{1,2} Such rearrangements in leukemias play a primary role in establishment and maintenance of malignancy^{3–7} and in induction of clinical remission by drugs that target activities of the encoded fusion oncoproteins.^{5,7,8} Gene rearrangements are now used commonly in the molecular diagnosis of cancer.

Aneuploidy is another frequent genetic abnormality in cancer. It results from full or partial aneusomies in which the copy number of entire chromosomes or chromosomal subregions is altered. Aneuploidy per se has been proposed to be an early and genetically destabilizing force in cancer development.^{9–13} Increasing aneuploidy is associated with cancer progression, but the molecular mechanisms related to aneuploidy in cancer are poorly understood.

Chromosomal rearrangements at 3p25 have been reported in human tumors arising from thyroid follicular epithelial cells.^{14–22} One such rearrangement, t(2;3)(q13;p25), results in a *PAX8-PPAR γ* gene fusion,²¹ and is the predominant member of a family of *PPAR γ* rearrangements in follicular thyroid carcinoma.^{20,21} Even so, there

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is a striking disparity in the overall number of rearrangements that have been discovered in carcinomas compared to leukemias and sarcomas.² Two adult carcinomas, both arising within the thyroid gland, have been shown to harbor specific gene rearrangement families.^{20,21,23,24} Thus, the thyroid provides a tractable carcinoma model with which to investigate gene rearrangement mechanisms.

Here, we have used interphase fluorescence *in situ* hybridization (FISH) and immunohistochemistry to define the prevalence, specificity, and clinicopathological correlates of 3p25 genetic alterations in human follicular thyroid tumors. Our findings suggest that distinct genetic pathways exist in early follicular thyroid cancer and that *PPAR* γ rearrangements characterize a specific follicular carcinoma entity. The results highlight the thyroid as a natural cancer model with which to investigate both gene rearrangement and aneuploidy mechanisms in epithelial malignancy.

Materials and Methods

Pathological Diagnoses

We retrieved pathology materials from all thyroidectomy specimens diagnosed with follicular thyroid carcinoma at Brigham and Women's Hospital (BWH), Boston, MA, in the 13-year period 1988 to 2000. Original pathological diagnoses were rendered by individual staff pathologists at BWH. More than 40% of cases had been reviewed by multiple BWH pathologists and ~15% had been reviewed by outside pathologists in expert consultations. All cases were re-examined independently and blindly with respect to molecular and clinical features by three pathologists (TGK, CAF, and VN) using current morphological criteria.^{25–27} Four follicular carcinomas were reclassified as papillary carcinomas because well-developed nuclear clearing, grooves, and/or focal papillary architecture were seen. Seven follicular carcinomas were reclassified as follicular adenomas because unequivocal capsular penetration or vascular invasion was not present. The remaining 42 follicular carcinomas and additional study specimens, including 90 papillary carcinomas, 40 follicular adenomas, 15 Hurthle cell carcinomas, and 10 multinodular goiters from BWH, and 15 Hurthle cell carcinomas from the Massachusetts General Hospital (MGH), were included in our final series. Some features of the 6 follicular carcinomas, 20 follicular adenomas, 10 papillary carcinomas, and 10 multinodular goiters have been reported previously.²¹ The work was approved by the joint BWH/MGH institutional review board.

Cytogenetics and FISH

Karyotyping was performed on Giemsa-banded metaphase spreads as described.²⁸ Formalin-fixed, paraffin-embedded sections were prepared for FISH according to a procedure modified from Bull and Harnden.²⁹ Briefly, 3- to 4- μ m sections were deparaffinized in xylene and mi-

crowaved for 30 to 60 minutes at 98°C in 100 mmol/L of Tris and 50 mmol/L of disodium ethylenediaminetetraacetic acid, pH 8. The tissue was digested with Digest-All III pepsin (Zymed, South San Francisco, CA) two times at 37°C (15 to 30 minutes each), washed, and postfixed in 10% phosphate-buffered formalin for 1 minute. Slides were denatured at 94°C for 3 to 5 minutes with biotin- and digoxigenin-labeled 3p25 YAC probes (753f7 and 932f3),²¹ incubated for 18 to 48 hours at 37°C, washed at 72°C in 0.5 $\times$ standard saline citrate for 5 minutes, and incubated with fluorescein isothiocyanate-anti-digoxigenin (Roche, Indianapolis, IN) and 594 rhodamine streptavidin (Molecular Probes, Eugene, OR) detection reagents in CAS block (Zymed).

Immunohistochemistry

Immunohistochemistry was performed using the monoclonal antibody E8 raised against a human *PPAR* γ synthetic peptide antigen (Santa Cruz Biotechnology, Santa Cruz, CA). E8 (1:300) was incubated overnight at 4°C on paraffin-embedded tissue sections after microwave antigen retrieval (10 mmol/L citrate buffer, pH 6, at 98°C for 30 minutes). Immune complexes were detected with the ENVISION nonbiotin system (DAKO, Carpinteria, CA) to circumvent known endogenous thyroid biotin-like activities. Controls for antibody specificity included preincubation of E8 with blocking synthetic peptide (Santa Cruz Biotechnology) and exclusion of E8 on tumor and normal thyroid sections. Positive and negative control tissue sections (containing or lacking *PPAR* γ rearrangement by molecular analyses) were included in each run. Consistent, diffuse *PPAR* γ nuclear immunoreactivity in tumor relative to normal thyroid tissues in the same sections was scored into three categories for comparative purposes: category 1, immunoreactivity in tumor nuclei comparable or less than that in normal thyroid nuclei; category 2, immunoreactivity in tumor nuclei elevated mildly over normal thyroid nuclei; category 3, immunoreactivity in tumor nuclei elevated highly over normal thyroid nuclei. Only category 3 tumors had *PPAR* γ rearrangement. Focal *PPAR* γ nuclear immunoreactivity, seen rarely in small cell clusters and microfollicles within tumors or reactive thyrocytes, and cytoplasmic *PPAR* γ immunoreactivity, which was generally low, focal, and variable, were disregarded in our analyses.

Clinical and Pathological Features and Statistical Analyses

Tumor pathology data and clinical preoperative and follow-up data were obtained by report and chart reviews and by contact with the patient's primary endocrinologist and/or primary care physician. Values for continuous variables were calculated as the mean $\pm$ SD, and values for categorical variables were calculated as percentages. Statistical differences were assessed using the Student's *t*-test for continuous data and the chi-square or Fisher's exact test for categorical data. All tests were two-tailed. Tumor capsular thickness was scored subjec-

A. 3p25 chromosomal translocation

B. 3p25/PPAR γ gene rearrangement

C. 3p25 aneusomy

Figure 1. 3p25 genetic abnormalities in follicular thyroid carcinoma. **A:** Chromosomal rearrangements such as t(2;3)(q13;p25) (**black arrowheads**) are present in a subset of follicular thyroid carcinomas. **B:** A 3p25 FISH assay with DNA probes flanking the *PPAR γ* gene demonstrates 3p25 rearrangement (**white arrowheads**) in a paraffin-embedded follicular thyroid carcinoma. **C:** The FISH assay also detects 3p25 aneusomy (tetrasomy in this tumor) in the presence or absence of 3p25 rearrangement.

tively as mild, moderate, or thick and objectively using a stage micrometer. Neither method yielded a statistical difference between tumors with or without *PPAR γ* alterations.

Results

Detection of 3p25/PPAR γ Rearrangement and 3p25 Aneusomy

The single follicular thyroid carcinoma karyotyped in our series contained t(2;3)(q13;p25) (Figure 1A, black arrowheads), a chromosomal rearrangement identified previ-

ously in follicular thyroid tumors.^{15,17–19,21,22} Recent studies have shown that t(2;3)(q13;p25) forms a *PAX8-PPAR γ* gene fusion²¹ that is present in a subset of follicular carcinomas.^{21,30,31} Here, we used interphase FISH with probes flanking the *PPAR γ* gene to investigate these 3p25 rearrangements.

3p25 rearrangements were observed by FISH in 11 of 42 (26%) low-stage follicular carcinomas, 0 of 40 follicular adenomas, 1 of 30 Hurthle cell carcinomas, 1 of 90 papillary carcinomas, and 0 of 10 multinodular goiters (Figure 1B, Table 1). The FISH assay also detected 3p25 genetic imbalances (3p25 aneusomy) in 12 of 42 (29%) follicular thyroid carcinomas (Figure 1C, Table 1). 3p25 aneusomy consisted of trisomy (five cases), trisomy with focal monosomy (one case), and tetrasomy (six cases). No follicular carcinoma had both 3p25 aneusomy and 3p25 rearrangement ($P = 0.01$). In total, 23 of 42 (55%) follicular thyroid carcinomas had 3p25 genetic alterations.

Detection of PPAR γ Fusion Protein by Immunohistochemistry

Strong, diffuse nuclear immunoreactivity for *PPAR γ* was observed in all 11 follicular thyroid carcinomas with 3p25 rearrangement but not in normal thyroid tissues or thyroid tumors such as follicular adenomas without *PPAR γ* rearrangement (Figure 2A). On the other hand, 11 of 12 follicular carcinomas with 3p25 aneusomy exhibited no nuclear *PPAR γ* immunoreactivity (Figure 2A). Mildly elevated nuclear *PPAR γ* immunoreactivity relative to normal thyroid tissue was seen in a small subset (6 of 42) of follicular carcinomas without *PPAR γ* rearrangement (one with 3p25 aneusomy). Cytoplasmic *PPAR γ* immunoreactivity was infrequent, low, and focal in both tumor and normal thyroid tissues and was disregarded in our analyses. In summary, 18 of 42 (43%) follicular thyroid carcinomas had elevated nuclear *PPAR γ* expression. Strong, diffuse nuclear *PPAR γ* expression was observed in all 11 cases with 3p25 rearrangement.

Clinical and Pathological Correlations in Follicular Carcinoma Patients

We compared the clinical and pathological features of patients with follicular thyroid carcinomas containing or lacking 3p25/PPAR γ rearrangement and 3p25 aneusomy. Clinical features that did not differ statistically ($P > 0.05$) in these groups included patient sex, age at diagnosis, mean tumor size, TMN stage at diagnosis (98% stage I or II), and frequency of regional lymph node spread (Table 1). Follicular carcinoma patients with *PPAR γ* rearrangement had previous nonthyroid cancers more frequently than follicular carcinoma patients without *PPAR γ* rearrangement ($P < 0.01$, Table 1). One patient (36 years of age) had Hodgkin's disease and a carcinoid tumor metastatic to lung. A second patient (25 years of age) had a testicular germ cell cancer. A third patient (36 years of age) had Hodgkin's disease and a lung adeno-

Table 1. Clinical and Pathologic Features of Follicular Carcinoma Patients with 3p25/PPAR γ Rearrangement and 3p25 Aneusomy

	All follicular carcinomas	Follicular carcinomas with 3p25/PPAR γ rearrangement	Follicular carcinomas without 3p25/PPAR γ rearrangement	<i>P</i> value	Follicular carcinomas with 3p25 aneusomy	Follicular carcinomas without 3p25 aneusomy	<i>P</i> value
Clinical features							
Number of patients	42 (100%)	11 (26%)	31 (74%)		12 (29%)	30 (71%)	
Percent female	77	82	81	0.93	92	73	0.25
Age at diagnosis (years)	40.6 $\pm$ 16.5	35.6 $\pm$ 9.7	42.4 $\pm$ 18.1	0.25	46.5 $\pm$ 24.3	38.5 $\pm$ 11.8	0.15
Tumor size (cm)	3.5 $\pm$ 1.8	2.8 $\pm$ 0.7	3.8 $\pm$ 2.0	0.17	4.0 $\pm$ 2.6	3.3 $\pm$ 1.4	0.25
TNM stage at diagnosis							
I	67%	82%	62%	0.62	59%	70%	0.1
II	29%	18%	32%		25%	30%	
III	2%	0%	3%		8%	0%	
IV	2%	0%	3%		8%	0%	
Lymph node involvement							
Positive	0%	0%	3%		8%	0%	
Negative	100%	100%	97%		92%	100%	
Prior nonthyroid cancer	3 (7%)	3 (27%)	0%	<0.01	0%	3%	<0.01
Pathologic features							
Vascular invasion	21 (50%)	9 (82%)	12 (39%)	0.01	5 (42%)	16 (53%)	0.5
Solid/nested histology	22 (52%)	11 (100%)	11 (35%)	<0.001	4 (33%)	21 (70%)	0.04
Capsular invasion	38 (90%)	11 (100%)	27 (87%)	0.21	10 (83%)	28 (93%)	0.56
Capsular penetration	30 (71%)	7 (64%)	23 (74%)	0.51	8 (67%)	22 (73%)	0.72
Thick tumor capsule	18 (43%)	6 (55%)	11 (35%)	0.27	3 (25%)	15 (50%)	0.18

carcinoma. Most follicular carcinoma patients received postsurgical radio-iodine ablation and suppressive thyroxine therapy. Follow-up intervals were statistically similar for patients with (mean, 5.7 years; 9 of 11 patients) or without (mean, 5.3 years; 20 of 31 patients) PPAR γ rearrangement and patients with (mean, 3.9 years; 8 of 12 patients) or without (mean, 5.7 years; 20 of 30 patients) 3p25 aneusomy. No differences in thyroid cancer recurrence, progression, or mortality were noted in these groups.

Pathological features of follicular carcinomas in patients with PPAR γ rearrangement differed significantly from those without PPAR γ rearrangement. Most follicular carcinomas with PPAR γ rearrangement were unilateral, encapsulated tumors that exhibited vascular invasion ($P = 0.01$) and well-defined areas of solid/nested tumor histology ($P < 0.001$; Figure 2B) compared with follicular carcinomas without PPAR γ rearrangement (Table 1). Follicular carcinomas with PPAR γ rearrangement lacked 3p25 aneusomy ($P = 0.01$) but were similar to follicular carcinomas without PPAR γ rearrangement in capsular invasion ($P = 0.21$), capsular penetration ($P = 0.51$), and capsular thickness ($P = 0.27$; Table 1). Follicular carcinomas with 3p25 aneusomy less frequently exhibited solid/nested tumor histology than follicular carcinomas with PPAR γ rearrangement ($P = 0.04$; Table 1).

Discussion

Our experiments provide evidence that PPAR γ gene rearrangement and 3p25 aneusomy arise independently in early follicular thyroid carcinoma subgroups. Twenty-three of 42 (55%) follicular carcinomas had either PPAR γ rearrangement or 3p25 aneusomy but none had both. Gene rearrangements are pathogenic aberrations in many blood cell cancers,^{2,32} in which they are early and

perhaps even initiating events. PPAR γ rearrangements seem to provide this type of oncogenic stimulus in follicular carcinomas.²¹ Aneuploidy is another early genetic alteration in cancer and it may promote genetic instability.^{9,33} Our findings suggest that PPAR γ rearrangement and aneuploidy have an either/or relationship in early follicular carcinomas and that they are independent genetic events in separate pathways of thyroid tumorigenesis. RAS mutations, other early genetic alterations in follicular carcinoma,^{34–36} also appear to arise independently of PPAR γ rearrangement (M. Nikiforova, R. Lynch, P. Biddinger, E. Alexander, G. Dorn, G. Tallini, T. Kroll, and Y. Nikiforov, unpublished data). Moreover, papillary carcinomas (thought to arise from the same follicular cells as follicular carcinomas) frequently have rearrangements of the RET^{37–39} or NTRK1^{40,41} genes. Thus, various early genetic aberrations appear to identify alternative oncogenic pathways within the thyroid. This pattern appears different from the single pathway of step-wise genetic progression envisioned for colorectal and pancreatic carcinomas, which nearly all contain APC^{10,42,43} or KRAS⁴⁴ mutations, respectively.

Although PPAR γ rearrangement and aneuploidy appear to arise independently in early follicular carcinomas, they can co-exist^{16,18} (TG Kroll and JA Fletcher, unpublished data). In two such published cases, PPAR γ rearrangement and aneuploidy appeared together in recurrent¹⁶ and metastatic¹⁸ follicular cancers. Hence, it is possible that PPAR γ rearrangement, aneuploidy, and other genetic alterations are rarely associated in low-stage tumors but can be acquired sequentially to create follicular cancers with more aggressive clinical behavior.

Considering the overall data, it seems most appropriate to consider follicular carcinomas with PPAR γ rearrangement a distinct thyroid cancer entity. Characteristic pathological features of this entity appear to be vascular

A. $PPAR\gamma$ immunohistochemistry

B. Solid/nested tumor histology

Figure 2. Follicular thyroid carcinomas with 3p25 genetic alterations. **A:** Follicular thyroid carcinomas (FC) with $PPAR\gamma$ gene rearrangement exhibit strong, diffuse $PPAR\gamma$ nuclear immunoreactivity as the result of $PPAR\gamma$ fusion protein expression. In contrast, normal thyroid tissues and most thyroid tumors without $PPAR\gamma$ rearrangement exhibit little or no nuclear $PPAR\gamma$ immunoreactivity. **B:** Follicular thyroid carcinomas with $PPAR\gamma$ rearrangement (FC) tend to have vascular invasion (data not shown) and well-defined regions of solid/nested tumor histology shown here adjacent to microfollicular and trabecular areas of the tumor. All histologies in early follicular carcinomas with $PPAR\gamma$ rearrangement exhibit strong $PPAR\gamma$ nuclear immunoreactivity.

invasion ($P = 0.01$), areas of solid/nested tumor histology ($P < 0.001$), and the absence of 3p25 aneusomy ($P = 0.01$). Vascular invasion and capsular penetration are long used morphological criteria for pathological diagnosis of follicular carcinoma,^{27,45–49} and metastatic follicular carcinomas with $PPAR\gamma$ rearrangement have been observed¹⁸ (T. Dwight and C. Larsson, unpublished data). The solid/nested tumor histology is similar to that seen in poorly differentiated insular carcinomas^{50,51} except that appreciable necrosis or mitoses are not present. Analysis of higher stage cancers with longer clinical follow-up will be important to define the genetic and biological pathways of progression in follicular carcinomas with $PPAR\gamma$ rearrangement and aneuploidy.

Strong, diffuse nuclear immunoreactivity for $PPAR\gamma$ was observed in all 11 follicular carcinomas with 3p25 rearrangement, whereas 6 additional follicular carcinomas without 3p25 rearrangement (one with 3p25 aneusomy) exhibited mildly elevated $PPAR\gamma$ nuclear immunoreactivity. All remaining follicular carcinomas without $PPAR\gamma$ rearrangement exhibited no $PPAR\gamma$ nuclear expression. These observations suggest that multiple

mechanisms of $PPAR\gamma$ deregulation exist in thyroid cancer and this may explain, at least in part, the various growth effects of $PPAR\gamma$ ligands on thyroid cancer cell lines *in vitro*⁵² and *in vivo*.⁵³ Alternate mechanisms of $PPAR\gamma$ deregulation could include cryptic rearrangements, point mutations, or deletions that do not disrupt the physical relationship of our FISH probes or epigenetic/regulatory factors that affect $PPAR\gamma$ transcription, translation, or degradation.

An interesting clinical feature of follicular carcinoma patients with $PPAR\gamma$ rearrangement was an apparent increase in previous nonthyroid cancers ($P < 0.001$). The robustness of this correlation is uncertain because of low patient numbers but it raises the possibilities that $PPAR\gamma$ rearrangement might be associated with cancer predisposition and/or previous cancer therapy. All three patients had previous cancers treated commonly with irradiation, but we could document a definite history of irradiation only in one.

We detected $PPAR\gamma$ rearrangements in a significant fraction of follicular carcinomas but not other thyroid tumors, supporting their high specificity. $PPAR\gamma$ rearrangements were absent from most Hurthle cell carcinomas, supporting the idea that follicular and Hurthle cell carcinomas should be considered separate thyroid cancer classes. Although not observed in our series, some follicular adenomas with t(2,3)(q13;p25)/PAX8- $PPAR\gamma$ have been reported.^{14,15,17,19,31} We suggest that these morphological follicular adenomas are best considered early *in situ* follicular carcinomas with malignant potential. Several observations are compatible with the possibility. First, gene rearrangements involving transcription factors are early alterations in many blood cell and connective tissue malignancies and their detection (even by polymerase chain reaction) often defines malignancy. Secondly, the majority of thyroid tumors so far shown to have $PPAR\gamma$ rearrangements are bonafide follicular carcinomas with vascular invasion, our best pathological indicator of malignancy in absence of local-regional spread or metastases. Advanced stage follicular carcinomas with $PPAR\gamma$ rearrangement have been observed^{16,18} (T. Dwight and C. Larsson, unpublished data). Thirdly, a clonal alteration such as $PPAR\gamma$ rearrangement is expected in some morphological follicular adenomas in which vascular invasion and capsular penetration are not yet developed or are missed by standard histological sectioning. Fourth, the high percentage of follicular carcinoma cells containing $PPAR\gamma$ rearrangements and the gross pathology patterns argue against follicular carcinomas arising often from benign thyroid (adenomatous) nodules. Finally, cytogenetic,^{14–16,18–20,54,55} comparative genomic hybridization,^{56,57} and loss of heterozygosity^{58–61} studies suggest that follicular adenomas and carcinomas are overall genetically distinct thyroid tumor groups.

We have also observed at low frequency papillary thyroid carcinomas with $PPAR\gamma$ rearrangement and expect more to be identified.²² It is therefore noteworthy that a minority (3 of 11) of our follicular carcinomas with $PPAR\gamma$ rearrangement exhibited identifiable but incompletely developed papillary carcinoma-like nuclear

changes (clearing and focal grooves). However, all three tumors with such features were encapsulated follicular-patterned lesions with vascular invasion—classic follicular carcinoma characteristics. None were felt to be suspicious for papillary carcinoma on preoperative cytology in which such nuclear changes are most sensitive and specific for papillary carcinoma. Even so, these observations suggest that follicular carcinomas with *PPAR* γ rearrangements do exhibit some morphological variation.

Well-differentiated follicular thyroid tumors with mixtures of papillary, follicular, and/or Hurthle cell morphologies are not uncommon^{62–64} and they highlight an overlapping morphological spectra that confounds attempts to diagnose follicular-patterned thyroid lesions in a precise and accurate way.^{62,65,66} This histological overlap among thyroid carcinoma subtypes poses challenges in evaluating molecular genetic correlates. *RET* rearrangements were originally thought specific for papillary thyroid carcinoma,^{67,68} but have recently been identified in up to 50% of benign and malignant Hurthle cell tumors.^{69–71} Apparently identical *RET* rearrangements have also been demonstrated in follicular thyroid tumors with very different morphological and biological patterns.^{72–77} These observations suggest that multiple genetic and/or epigenetic events collaborate to determine overall thyroid cancer phenotype.⁷⁸ It seems likely that such alterations will ultimately prove useful in classifying thyroid tumors into more definitive biological and clinical subgroups.

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