ORIGINAL ARTICLE

Trefoil Factor 3 Immunohistochemical Characterization of Follicular Thyroid Lesions From Tissue Microarray

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Objectives: To characterize trefoil factor 3 (TFF3) expression in normal thyroid tissue samples compared with that in follicular adenoma, follicular carcinoma, and follicular variant of papillary thyroid carcinoma using immunohistochemistry on tissue microarrays.

Design: Immunohistochemical analysis of 83 normal thyroid tissue and of 83 follicular neoplasms (26 follicular adenomas, 25 follicular variant of papillary thyroid carcinoma, 23 follicular thyroid carcinomas, and 9 papillary thyroid carcinomas) was performed using an antibody to TFF3 on tissue microarray sections composed of formalin-fixed, paraffin-embedded tissue samples.

Setting: Academic research.

Patients: Thyroid tissue samples collected from patients over a 15-year period were obtained from the University of North Carolina Hospitals Division of Surgical Pathology archives.

Main Outcome Measures: Thyroid tissue samples were graded by a pathologist based on intensity of antibody staining and on percentage of cells stained. Localization of TFF3 antibody was noted. Data were analyzed for semiquantitative differences in immunohistochemical intensity of antibody staining and in percentage of cells stained among normal thyroid tissue samples, follicular adenoma, follicular thyroid carcinoma, follicular variant of papillary thyroid carcinoma, and papillary thyroid carcinoma.

Results: Semiquantitative analysis demonstrated that immunohistochemistry detects significant levels of TFF3 expression in normal thyroid tissue samples compared with that in follicular lesions based on intensity of antibody staining ($P<.05$). Only follicular thyroid carcinoma demonstrated a significant reduction in percentage of cells stained compared with that in normal thyroid tissue samples ($P=.03$). No significant differences in intensity of antibody staining or in the percentage of cells stained were noted among follicular adenoma, follicular thyroid carcinoma, follicular variant of papillary thyroid carcinoma, or papillary thyroid carcinoma. Trefoil factor 3 staining localized to the cytoplasm.

Conclusions: Protein expression data validate gene expression findings that follicular neoplastic lesions have decreased expression of TFF3 compared with that in normal thyroid tissue samples. These findings contribute to evidence suggesting that TFF3 may have a role in normal thyroid tissue function and that thyroid carcinomas may have reduced expression of TFF3, in contradistinction to other carcinomas that overexpress TFF3.

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The American Cancer Society[1] estimated that in 2008 there would be 37 340 new diagnoses of thyroid carcinoma, which constitutes more than 90% of all endocrine cancers and accounts for the highest annual mortality from endocrine cancer.[2] The incidence of thyroid cancer has been reported to be increasing at a rate of 3% per year,[3] and patients are diagnosed from a much larger group of patients who are initially seen with thyroid nodules investigated on fine-needle aspiration biopsy. For approximately 30 years, fine-needle aspiration has been the least invasive and most efficient initial test for evaluation of solitary thyroid nodules. Although fine-needle aspiration excels at identifying papillary thyroid carcinoma (PTC), a major deficiency of this diagnostic procedure is that follicular adenoma (FA), follicular thyroid carcinoma (FTC), and follicular variant of PTC (FVPTC) cannot be differentiated cytopathologically.[4] The morphologic features of follicular lesions exhibit varying degrees of

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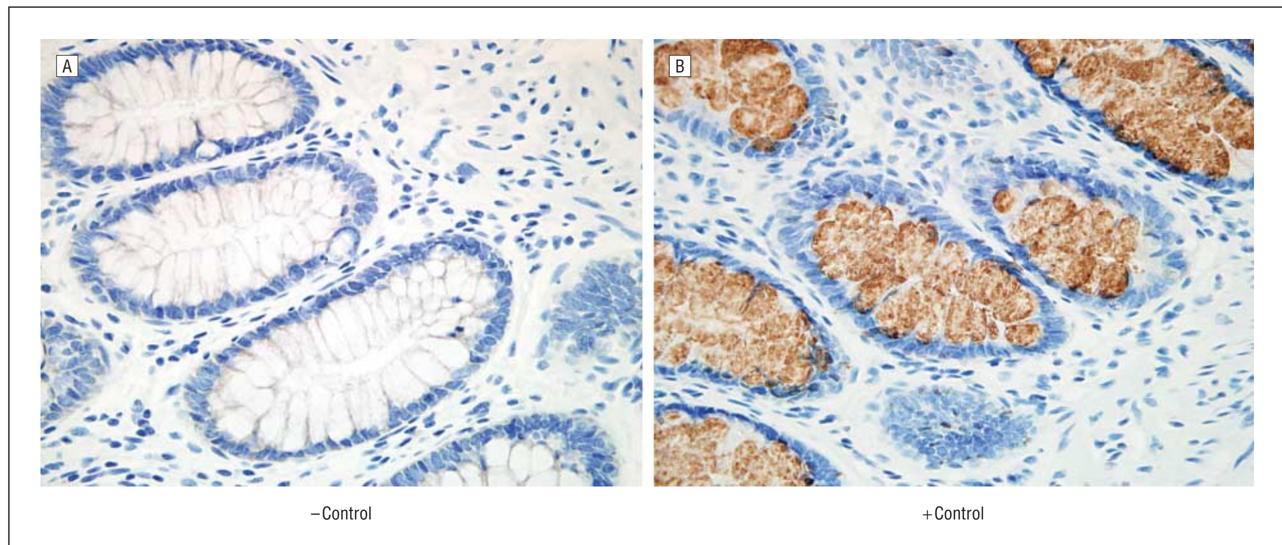

Figure 1. Colon tissue sample at high power (original magnification ×40). A, Negative control shows absence of staining when IgG1 is used as the primary antibody. B, Positive control shows cytoplasmic staining after incubation with trefoil factor 3 antibody.

cellular atypia, and the diagnosis of follicular carcinoma is dependent on the finding of capsular or vascular invasion on formal pathologic evaluation.

Human intestinal trefoil factor 3 (*TFF3* [NCBI Entrez Gene_7033]) is a differentially expressed gene between FA and carcinomas[5] and was previously shown to be differentially expressed between normal thyroid tissue and PTCs.[6] The *TFF3* gene product is primarily expressed in the intestine, specifically the goblet cells and the gland acini, as well as the distal ducts of Brunner glands.[7,8] Trefoil factors are a class of soluble protein with a characteristic tree-looped trefoil structure formed through interchain disulfide bonding.[9] They are packaged in the Golgi apparatus into mucous granules and are secreted with mucins to enhance the protective gastrointestinal mucosa layer.[8,10]

Although immunohistochemical analysis of TFF3 has been performed in colon and prostate,[11] expression has not been characterized in thyroid tissue. A review of the literature highlights messenger RNA (mRNA) differences between FA and carcinoma, with 2-fold increased *TFF3* mRNA expression in FA vs FTC ($P = .002$),[5] but differences in protein expression on immunohistochemistry (IHC) have not been reported. In this study, FA, FTC, PTC, and FVPTC were analyzed using IHC with antibodies targeting TFF3 to validate that differences in gene transcription translate to variations in protein expression among follicular lesions.

METHODS

Formalin-fixed, paraffin-embedded thyroid tissue samples collected over a 15-year period were obtained from the University of North Carolina Hospitals Division of Surgical Pathology archives. Approval to obtain the tissue samples was granted by the University of North Carolina Institutional Review Board. Tumor samples were obtained in duplicate from 30 FAs, 30 FTCs, 29 FVPTCs, and 11 PTCs. Adjacent normal thyroid tissue from each specimen was also evaluated. Specimens with more than 1 diagnosis or histologic finding (such as Hurthle cell changes) were excluded. Corresponding hematoxylin-eosin–stained sections of the tissue samples were obtained and were reviewed by a pathologist (L.B.T.) for confirmation of diagnosis.

TISSUE MICROARRAY CONSTRUCTION

Tissue microarrays (TMAs) were constructed by the University of North Carolina Anatomic Pathology Core Facility. The hematoxylin-eosin–stained slides of thyroid specimens were reviewed by a surgical pathologist (L.B.T.) uninvolved in the initial diagnoses of the selected tissue samples, who confirmed the diagnoses established by the Division of Surgical Pathology before outlining areas of normal and tumor tissue samples for TMA cores. A spreadsheet was constructed using sample accession numbers that excluded patient information and pathologic diagnoses to allow for blinded grading by the pathologists (C.F.H. and L.B.T.). Using a manual tissue arrayer (MTA-1; Beecher Instruments Inc, Sun Prairie, Wisconsin), 1-mm cores of matched normal and tumor thyroid tissue samples were punched from the surgical pathology (donor) block and were placed in the recipient block. The block was heated to congeal the samples into the block, and a paraffin layer was applied for proper facing.

IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical staining for TFF3 was performed on 4-μm tissue array sections. Using a kit (Cytomation LSAB plus; DAKO North America, Inc, Carpenteria, California), TMA slides were heated at 60°C for 1 hour and then deparaffinized and dehydrated. After rinsing in automation buffer (DAKO North America, Inc), the slides were subjected to antigen retrieval using 1× citra buffer (6.0 pH; DAKO North America, Inc) at 100 C° (steam) for 30 minutes and were allowed to cool to room temperature. The slides were subjected to a serum-free protein block, a peroxidase block, and an avidin-biotin system block before incubation with TFF3 (mouse monoclonal antibody, CalBiochem, San Diego, California) at 1:50 dilution overnight at 4°C in a humidified chamber. Normal colon tissue, known to express TFF3, was used as a positive control (**Figure 1**). A nonspecific negative control consisted of normal colon tissue incubated with a standard negative control antibody (mouse monoclonal negative control IgG1 antibody; DAKO North America, Inc) at 1:50 dilution. A universal secondary antibody and streptavidin–horseradish peroxidase (LSAB2 kit,

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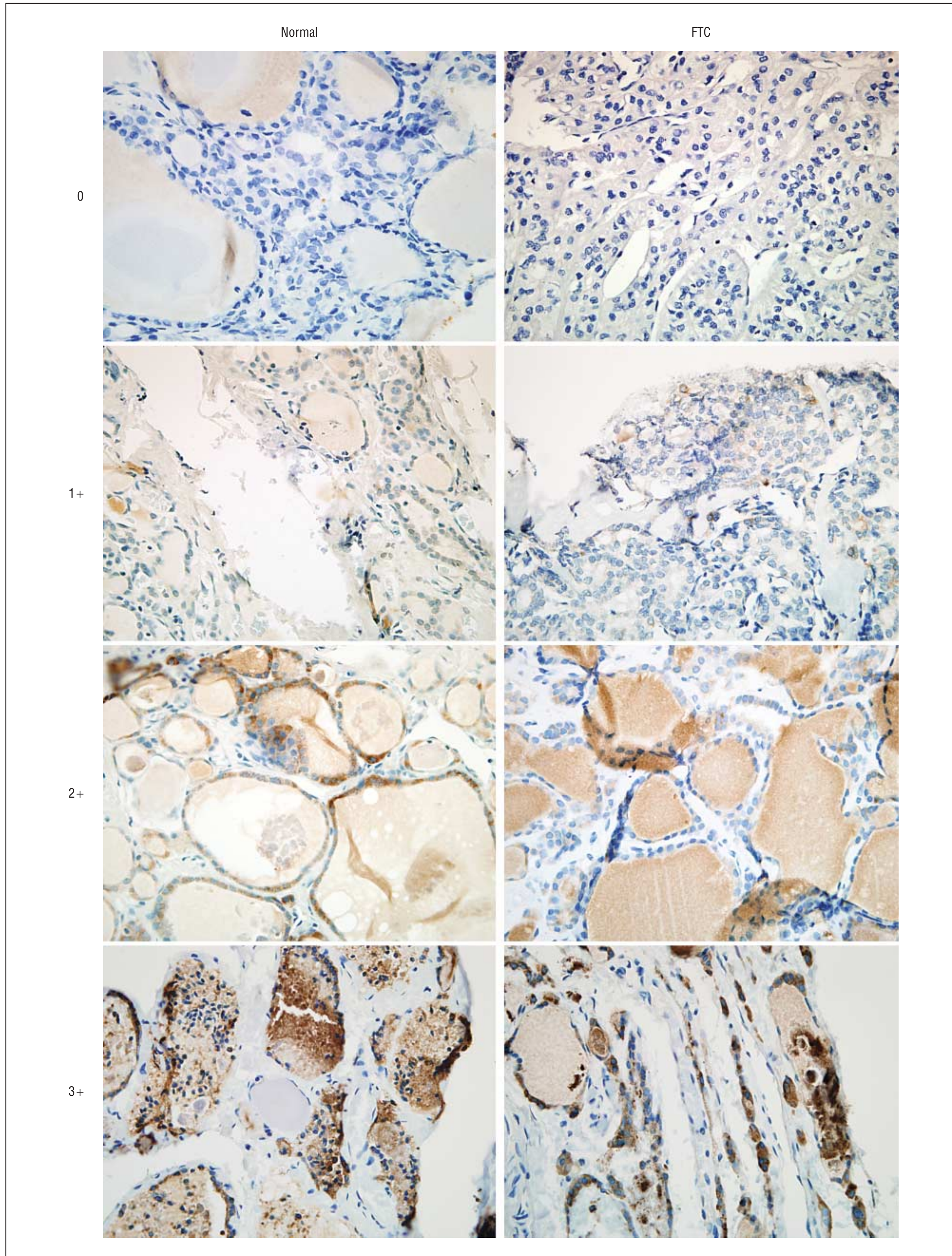

Figure 2. Normal thyroid tissue sample and follicular thyroid carcinoma (FTC) stained with trefoil factor 3 antibody at high power (original magnification ×40). Representative examples demonstrating scores for intensity of antibody staining from randomly selected normal and FTC tissue microarrays are shown. The percentages of cells stained vary as follows (from top left to right): 0%, 0%, 30%, 100%, 50%, 30%, 70%, and 50%). Trefoil factor 3 staining is localized to the cytoplasmic region.

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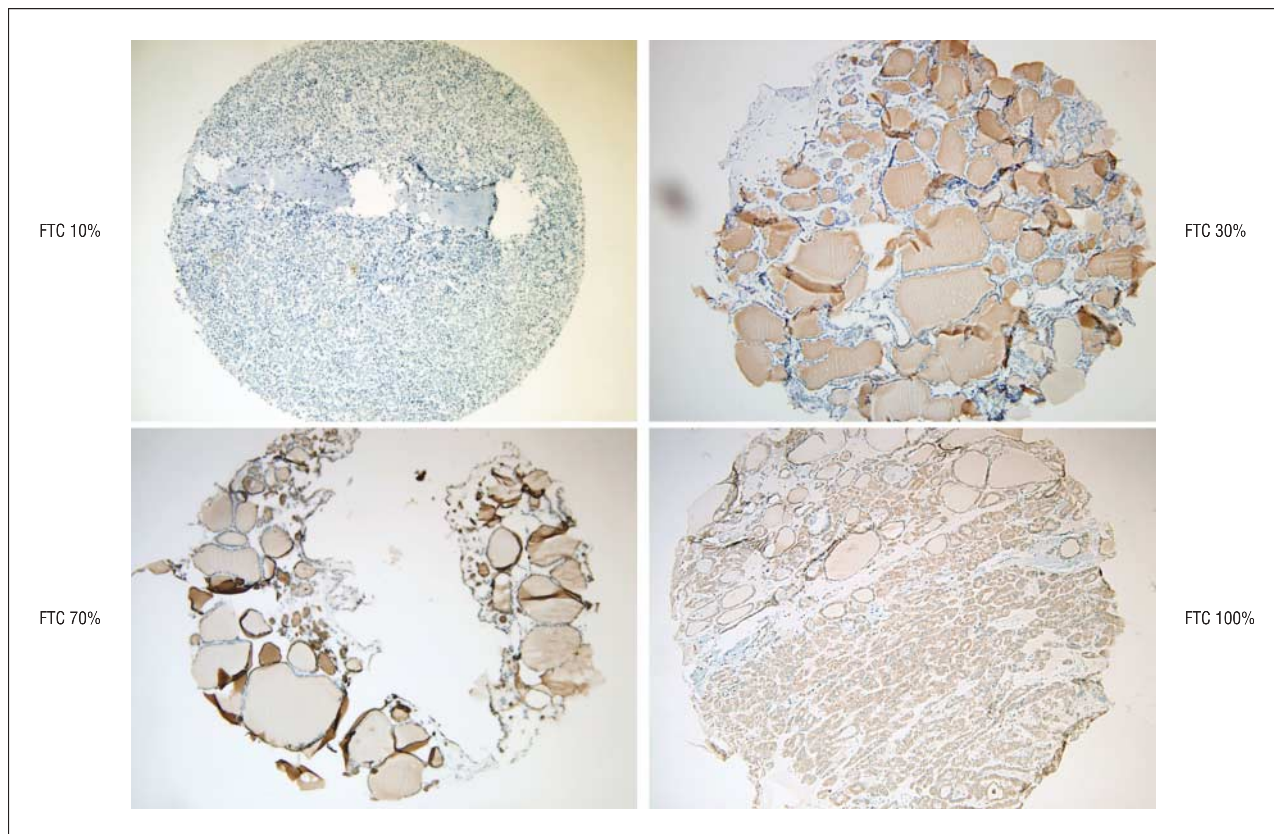

Figure 3. Follicular thyroid carcinoma (FTC) tissue microarray cores stained with trefoil factor 3 viewed at low power (original magnification ×10). Representative examples demonstrating scores for percentage of cells stained are shown. Each of 4 FTCs was scored 2+ on intensity of antibody staining.

DAKO North America, Inc) was applied to each slide after several washes with buffer. The TFF3 antibody was visualized using 3,3-diaminobenzidine at room temperature for 5 minutes. The slides were counterstained with hematoxylin, dehydrated, cleared with xylene, and coverslipped using a mounting medium (Permount; Fisher Science, San Francisco, California).

TMA SCORING

Slides were scored by a surgical pathologist (C.F.H. or L.B.T.) who was blinded to the arrangement of specimens within the TMA. The surgical pathologist scored the specimens using a predetermined set of criteria for cellular intensity of antibody staining (0, 1+, 2+, and 3+), staining pattern (cytoplasmic, membranous, or nuclear), and percentage of tumor and nontumor cells staining in relation to the entire core sample of the TMA (0% through 100% in increments of 10%). Percentages of cells stained were then placed in quartiles using the following scale: 1% to 25%, 26% to 50%, 51% to 75%, and 76% to 100% (**Figure 2** and **Figure 3** show examples of scoring). Scores of "quantity not sufficient" were assigned to positions on the TMA that had a scarcity of tissue such that it was impossible to assign a score; therefore, quantity-not-sufficient samples did not contribute to data analysis. Statistical analysis was performed on intensity of antibody staining and on percentage of stained cells.

STATISTICAL ANALYSIS

Exclusion of quantity-not-sufficient samples resulted in 26 FAs, 25 FVPTCs, 23 FTCs, and 9 PTCs. Because duplicate samples were obtained, the scores were averaged for the statistical analysis. For each type of follicular lesion, intensity of antibody staining and percentage of cells stained were compared between normal and tumor tissue samples (FA, FTC, PTC, or FVPTC) using the Wilcoxon signed rank test. The Kruskal-Wallis test was then performed to determine whether there was an overall difference between the scores for the 4 types of lesions.

RESULTS

The results of immunohistochemical staining with TFF3 in normal thyroid tissue samples are summarized in **Table 1**. All normal tissue samples stained positive for TFF3. Seventy-seven percent stained with an intensity of at least 2, with 42.2% of samples demonstrating greater than 50% staining. Positive TFF3 immunohistochemical staining was observed in 21 of 26 FAs (80.8%). Thirty-one percent stained with an intensity of at least 2, and 42.3% of FAs demonstrated greater than 50% staining. Follicular thyroid carcinomas stained positive for TFF3 in 21 of 23 samples (91.3%). Seventeen percent stained with an intensity of at least 2, and 34.7% of FTCs demonstrated greater than 50% staining.

Twenty-two follicular variants (88.0%) of PTCs stained positive for TFF3. Twenty-eight percent of these tissue samples stained with an intensity exceeding 2+, with 44.0% of samples demonstrating greater than 50% staining. Papillary thyroid carcinomas stained positive for TFF3 in all 9 samples, one of which stained with an intensity exceeding 2+. Greater than 50% staining was observed in 3 PTCs (33.3%). For all samples, TFF3 staining was evident only in the cytoplasm.

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Table 1. Immunohistochemical Expression of Trefoil Factor 3

Tissue Sample	Score for Intensity of Antibody Staining, No. (%)[a]							Total
	3	2.5	2	1.5	1	0.5	0	
Normal	4 (4.8)	8 (9.6)	52 (62.7)	6 (7.2)	13 (15.7)	0	0	**83**
FA	1 (3.8)	0	7 (26.9)	1 (3.8)	9 (34.6)	3 (11.5)	5 (19.2)	**26**
FTC	0	1 (4.3)	3 (13.0)	4 (17.4)	9 (39.1)	4 (17.4)	2 (8.7)	**23**
FVPTC	1 (4.0)	1 (4.0)	5 (20.0)	6 (24.0)	3 (12.0)	6 (24.0)	3 (12.0)	**25**
PTC	0	0	1 (11.1)	3 (33.3)	4 (44.4)	1 (11.1)	0	**9**

Tissue Sample	Score for Percentage of Cells Stained, No. (%)[a]									Total
	4	3.5	3	2.5	2	1.5	1	0.5	0	
Normal	14 (16.9)	8 (9.6)	13 (15.7)	15 (18.1)	20 (24.1)	10 (12.0)	3 (3.6)	0	0	**83**
FA	8 (30.8)	3 (11.5)	0	2 (7.7)	2 (7.7)	1 (3.8)	3 (11.5)	2 (7.7)	5 (19.2)	**26**
FTC	3 (13.0)	2 (8.7)	3 (13.0)	1 (4.3)	5 (21.7)	0	0	7 (30.4)	2 (8.7)	**23**
FVPTC	6 (24.0)	3 (12.0)	2 (8.0)	1 (4.0)	2 (8.0)	2 (8.0)	5 (20.0)	1 (4.0)	3 (12.0)	**25**
PTC	2 (22.0)	0	1 (11.1)	5 (55.6)	1 (11.1)	0	0	0	0	**9**

Abbreviations: FA, follicular adenoma; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; PTC, papillary thyroid carcinoma.
[a] Because of rounding, percentages do not total 100.

Table 2. Analysis of Immunohistochemical Expression of Trefoil Factor 3

Tissue Sample	Sample Size	Median Score		*P* Value
		Normal Tissue	Tumor Tissue	
Intensity of antibody staining				
FA	26	2	1	<.001
FTC	23	2	1	<.001
FVPTC	25	2	1.5	.02
PTC	9	2	1	.06
Percentage of cells stained				
FA	26	2.5	2.25	.12
FTC	23	2.5	2	.03
FVPTC	25	2.5	2	.33
PTC	9	2.5	2.5	.56

Abbreviations: FA, follicular adenoma; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; PTC, papillary thyroid carcinoma.

Table 2 summarizes the results of semiquantitative analyses of TFF3 immunohistochemical levels comparing normal thyroid tissue samples vs FA, FTC, PTC, and FVPTC. Significant differences ($P < .05$) in immunohistochemical staining intensity were seen among normal thyroid tissue samples and FA ($P < .001$), FTC ($P < .001$), and FVPTC ($P = .02$) and marginally for PTC ($P = .06$). Only FTC demonstrated a significant reduction in percentage of cells stained compared with that in normal thyroid tissue samples ($P = .03$). The overall test for any statistically significant differences in TFF3 staining intensity among FA, FTC, PTC, and FVPTC showed none ($P = .92$), and a similar result was seen for percentage of cells stained ($P = .46$).

COMMENT

The role of upregulated TFF3 in various carcinomas remains elusive, and its differential expression in thyroid tissue at the mRNA stage of protein synthesis is contrary to that of other carcinomas associated with TFF3. Aberrant *TFF3* gene expression is associated with gastrointestinal tract diseases, including Crohn disease, ulcerative colitis, and cholecysititis.[12-14] To our knowledge, there are no published results evaluating the expression of TFF3 in thyroid inflammatory conditions such as Hashimoto disease. Because Hashimoto disease results in destruction of normal follicular thyroid cells, it is plausible that TFF3 expression would decrease based on functional characteristics seen in the gastrointestinal system.

Various tissue carcinomas overexpress TFF3, including breast carcinoma, gastric carcinoma, colorectal carcinoma, and metastatic prostate cancers.[8] However, normal thyroid tissue, adenomatous goiter, and medullary carcinomas demonstrate abundant *TFF3* mRNA expression.[5,6] Each of these thyroid tissues produces hormones such as triiodothyronine, thyroxine, and calcitonin. Our study shows that TFF3 is localized to the cytoplasmic region of thyroid cells and correlates with expression findings in intestinal tissue, where TFF3 is secreted after packaging in the Golgi apparatus. The decreased protein expression of TFF3 in FA, FTC, and FVPTC may contribute to decreased hormone functionality in these tissue types. Although expression of *TFF3* mRNA has been shown to be decreased significantly in FTC compared with that in

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Table 3. Candidate Genes Identified as Potential Markers for Differentiating Follicular Thyroid Lesions

Source	Protein	Degree of mRNA Overexpression			Published Thyroid Immunohistochemistry Data Available
		Normal Tissue	Adenoma	Carcinoma	
Cerutti et al,[17] 2004	ITM1	5	0	27	Yes
	ARG2	2	0	21	Yes
Griffith et al,[18] 2006	MET	1	NQ	4.54	Yes
	TFF3	1	NQ	−22.04	No
Finley et al,[19] 2004	Galectin 3	1	1	3.5	Yes
	TFF3	NA	1	−3.8	No
Rodrigo et al,[20] 2006	CD44v6	NA	NQ	NQ	Yes
	HMGI	NA	NQ	NQ	Yes

Abbreviations: mRNA, messenger RNA; NA, normal thyroid tissue samples not quantified for amount of mRNA relative to follicular adenomas and carcinomas; NQ, reverse transcription–polymerase chain reaction products qualitatively detected without quantified differences reported.

FA,[5] our study shows no significant difference at the level of protein expression. This may be a function of the particular antibody used and the targeted epitope or a function of antibody staining variability in general.

Tissue samples that were obtained from the operating room were formalin fixed by standard protocol; however, there was no control for the duration of fixation, which may contribute to differences in intensity of antibody staining. In light of potential discrepancies in staining because of time differences in formalin fixation, we used 2 to 3+ as our cutoff for positive TFF3 staining. Our search for a standard immunohistochemical scoring system revealed that intensity scoring is inconsistent among pathologists when trying to distinguish 2+ and 3+.[15,16]

Seventy-four percent of FTCs analyzed with TFF3 antibody were described as minimally invasive FTCs in the pathology report (data not shown). Comparing 20 minimally invasive FTCs with FAs demonstrated no significant difference in IHC intensity scores ($P = .71$). There was insufficient evidence to show a significant difference in IHC intensity scores between 7 widely invasive FTCs and FAs ($P = .32$), although scores of widely invasive FTCs ranged from only 0.5 to 1, while FA scores ranged from 1 to 3.

Several articles and reviews have been recently published highlighting genes that are upregulated or downregulated and may serve as potential markers for distinguishing malignant follicular lesions (**Table 3**).[17-20] Most of the studies use reverse transcription–polymerase chain reaction combined with gene array analysis, and there is an increasing number of studies targeting expressed gene products using IHC. Although studies[17,21,22] have provided algorithms for detecting significant differences at the level of gene expression, mRNA evaluation using gene microarrays is technically impractical and remains cost prohibitive for the clinical setting. While IHC of fine-needle aspiration biopsies is more practical than diagnostic microarray, there are no reliable antibody markers or combinations of antibody markers to provide the sensitivity and specificity to guide clinical decision making.[23] Several gene products that demonstrate differential expression in FA vs FTC and FVPTC have been characterized using IHC; however, TFF3 has not been studied.[17,20,21,23]

Our study characterizes TFF3 protein expression using immunohistochemical staining in thyroid tissue and demonstrates decreased expression in FA, FTC, and FVPTC compared with that in normal thyroid tissue samples. Our observations correlate with findings that normal thyroid tissue abundantly expresses *TFF3* mRNA compared with that in FA, FTC, PTC, and FVPTC.[5,10] However, unlike in prior mRNA investigations, expression differences in FA vs FTC, PTC, and FVPTC were not apparent using IHC.

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Author Contributions: Dr Zanation had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Shores, Hart, and Zanation. *Acquisition of data:* Bryson, Hart, and Thorne. *Analysis and interpretation of data:* Patel and Deal. *Drafting of the manuscript:* Patel. *Critical revision of the manuscript for important intellectual content:* Bryson, Shores, Hart, Thorne, Deal, and Zanation. *Statistical analysis:* Deal. *Obtained funding:* Patel and Bryson. *Administrative, technical, and material support:* Shores, Hart, and Thorne. *Study supervision:* Zanation.

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