Human Pathology (2019) **91**, 36–42

ELSEVIER

Human PATHOLOGY

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Original contribution

Loss of nuclear localization of thyroid transcription factor 1 and adverse outcomes in papillary thyroid cancer☆,☆☆

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Received 1 April 2019; revised 10 June 2019; accepted 16 June 2019

Keywords:
TTF1;
Differentiation;
Extrathyroidal extension;
Lymph node metastases;
BRAF

Summary Function of the thyroid follicular cell depends on nuclear expression of thyroid transcription factor 1 (TTF1). Regulation of this key protein regulating iodide transport is not well known, but its loss is linked to the most lethal of thyroid malignancies. We examined TTF1 nuclear expression in the context of adverse pathological features, disease recurrence, and BRAF status in papillary thyroid carcinomas with (n = 182) and without (n = 303) nodal metastases. Overall nuclear expression level of TTF1 was strong and diffuse in approximately 73%, whereas 27% exhibited lower levels or a paucity of nuclear staining. In the same cohort, approximately 59% exhibited the *BRAF* mutation. On univariate analysis, low levels of TTF1 nuclear expression was linked to vascular invasion, extrathyroidal extension, and nodal metastases. Multivariate analysis indicated that low levels of TTF1 were most strongly linked to nodal metastases and vascular invasion. Interestingly, TTF1 levels were not linked to the *BRAF* mutation. TTF1 staining alone predicted disease recurrence, but when combined with BRAF status, the 2 markers exhibited a more marked influence. Patients lacking the *BRAF* mutation and exhibiting normal levels of TTF1 exhibited very low levels of disease recurrence (11% at 10 years). Conversely, patient tumors with low levels of TTF1 and the *BRAF* mutation recurred in 31% of cases in the same time frame. The mixed expression of BRAF under varying levels of differentiation may explain, in part, the contradictory studies regarding the impact of *BRAF* mutations on patient prognosis and also indicates a complex genomic signature for dedifferentiated thyroid cancer.

1. Introduction

Defining the need for radioactive iodine is a central element of risk stratification for papillary thyroid carcinoma [1,2]. Previous studies, incorporated and documented in the American Thyroid Association thyroid carcinoma guidelines, document important roles for tumor size and other pathologic features

☆ Competing interests: none.
☆☆ Funding/Support: This study was funded by a Canadian Institutes of Health Research Project Grant to Todd P. W. McMullen.
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https://doi.org/10.1016/j.humpath.2019.06.002
0046-8177/

when considering individual patients for therapy [3-6]. However, these morphologic and pathologic predictors can fail to predict resistance, and this is reflected in the wide variations in the application of radioactive iodine following thyroid surgery [7,8]. Clinical studies examining patient outcomes and the application of radioactive iodine or more advanced multitargeted kinase therapy still use morphologic assessments and long-term observation to map doubling times and growth patterns [9-12]. Ultimately, the pathways driving, or inhibiting, dedifferentiation are the key to understanding how individual patients will respond to therapy [13,14].

Transcription factors NKX2-1 (thyroid transcription factor 1 or TTF1) and paired-box gene 8 (PAX8) are absolutely required for thyroid follicular cell function [15]. Both transcription factors have often been defined as ubiquitous constituents of thyroid carcinomas and are sufficient and necessary for the transport of iodine. However, it is clear that the regulation of these transcription factors may be varied depending on disease severity [16,17]. Many efforts have been directed at treating dedifferentiation in thyroid cancer patients through modulation of mTOR, BRAF, ERK, AKT, and other pathways. More than a dozen case series and clinical trials have used individual pathway blockade or kinase inhibitors to attempt to restore differentiation that manifests clinically as radioactive iodine uptake [18,19]. These efforts have provided mixed results and significant toxicities; however, continued efforts with MEK inhibitors and BRAF inhibitors show promise for some patients [20].

The processes governing thyroglobulin production and dedifferentiation of thyroid carcinomas are not well understood despite the multitude of studies probing MEK pathway drivers [21,22]. Dedifferentiated tumors, lacking iodide transport and TTF1, could be treated potentially through forced expression as shown previous in model studies [23]. However, variations in TTF1 function cannot be accounted by assessments of *BRAF* or *RAS* mutations, and TTF1 mRNA levels do not closely correlate with aggressive disease [24-26]. Altered phosphorylation and cytoplasmic targeting of TTF1, with loss of nuclear TTF1 expression, may be an important mechanism underlying dedifferentiation and poor outcomes in papillary thyroid cancer [27]. Thus, immunohistochemical assessments of TTF1 may serve as a tool to examine the sum of mutational inputs that disrupt differentiation and potentially demark poor prognosis.

To identify the role of nuclear TTF1 localization in defining thyroid follicular cell differentiation and patient prognosis, we assessed the tissue array of more than 500 patients in a prospective database with follow-up varying from 1 to 528 months. The goal was to determine the subcellular abundance of this key mediator of differentiation in thyroid follicular cells. We examined the presence and absence of adverse pathologic features with respect to the level of nuclear TTF1 staining, as well as the application of radioactive iodine. We also examined if the presence or absence of *BRAF* or *ALK* mutations was linked to nuclear TTF1 expression and a moderator of tumor features and patient outcomes.

2. Materials and methods

2.1. Patients and tissues

This cohort study involved 525 consecutive patients with papillary thyroid cancer who underwent surgery from 1990 to 2012 at the University of Sydney Endocrine Surgery Unit. Cases were identified from a prospectively maintained thyroid surgery database after approval from the local institutional human research ethics committee. All cases of thyroid cancer in this cohort were treated with a total thyroidectomy in either 1 or 2 stages with nodal metastases addressed through selective neck dissections. The TTF1 analysis was performed under approval obtained through the Health Research Ethics Board of Alberta Cancer Committee HREBA.CC-16-0359. Paraffin-embedded tissue was available from the Department of Anatomical Pathology at the Royal North Shore Hospital, with approval from the Northern Sydney Local Health District ethics board. *Structural recurrence* was defined as disease that was visible on cross-sectional imaging (ultrasonography, computed tomography, magnetic resonance imaging, or positron emission tomography/computed tomography) and confirmed to be papillary thyroid carcinoma on cytology or histopathology, independent of serum thyroglobulin levels. All cases were reviewed centrally to confirm diagnosis, and tissue sampling was completed on representative samples best comprising the overall tumor histology. Less than 3% of this cohort comprised tall cell or columnar cell variants, and we did not perform a separate analysis on this group. Tissue microarrays were constructed from formalin-fixed, paraffin-embedded tissue containing 2 × 1–mm cores from each tumor. The classification of thyroid tumors was based on the World Health Organization criteria (2017).

2.2. Immunohistochemistry and scoring

Formalin-fixed, paraffin-embedded tissue sections of 4-μm thickness were deparaffinized and rehydrated. Evaluation of immunostaining was performed without knowledge of the clinical outcome, and all specimens had representative sections confirming that >90% of the specimen consisted of papillary thyroid carcinoma [28]. Sample cores on the tissue array that were fragmented or incomplete were not scored**.** Immunohistochemistry was performed using the clinically validated TTF1 antibody (NCL-L, Leica, Milton Keynes, UK) with antigen retrieval in citrate buffer for 48 minutes followed by staining with a 1/40 dilution of antibody. Previous analysis by our group and others using this protocol revealed good scoring reliability [27,28]. Cohort analysis by age of sample block revealed that TTF1 nuclear staining intensity did not vary significantly with respect specimen age ($P = .291$). *BRAF* V600E mutation-specific analysis was performed on all samples using a commercially available monoclonal antibody (clone VE1, Spring Bioscience, Pleasanton, CA) as outlined by Fraser et al in 2016 [29]. The ALK mutational

assessment on this cohort was completed previously using an ALK monoclonal antibody at 1:10 (clone 5A4, Novocastra, Leica Biosystems, Milton Keynes, UK) [30].

2.3. Statistical analysis

All statistical analyses were performed using the SPSS statistics version 15 (SPSS, Chicago, IL). Mean and standard error mean were presented for continuous variables, and frequency and percentages were reported for categorical variables. Scoring comparisons for the high and low cutoff between the 2 observers demonstrated complete agreement in approximately 88% of cases and a Cohen κ of 0.75 indicating substantial agreement. Variations in scoring were not linked to patient demographics or pathologic features. The correlations between 2 categorical variables were assessed using Pearson χ^2 tests. For cell frequencies less than 5, Fisher exact test was reported. Independent t tests were used to compare the mean values for total radioactive iodine activity applied. For all analyses, frequencies for the strong and moderate staining were combined and compared to that of the minimal staining. Correlations of continuous variables were tested using the Spearman ρ test because the data were non-normally distributed. $P < .05$ was considered for statistical significance. Classical 2 × 2 tables were used for estimating the diagnostic performance of markers. The calculated 95% confidence intervals were sufficient to establish the diagnostic superiority of one marker over the other. Recurrence-free survival was calculated from the day of treatment to the day of documented disease progression or recurrence. The logistic regression model was completed using TTF1 staining as the dependent variable adjusting for extrathyroidal extension, tumor stage, and nodal disease. Any patient who did not have an event was censored for the survival analyses. Kaplan-Meier estimates were obtained and presented along with their confidence intervals. Log-rank tests were used to compare the survival curves. Binary logistic regression was used to determine the factors associated with recurrence as the response variable.

3. Results

3.1. Demographics

We examined nuclear TTF1 expression in the tissue array with papillary thyroid cancer patients (n = 485) including 182 patients with nodal metastases and 9% of patients that underwent surgery for recurrent disease between 2 and 226 months. Overall, tumor size averaged 2.14 ± 1.66 cm with an average age of 47.8 ± 16.2 years, with the female to male ratio approximately 3:1. Fig. 1 exhibits representative sections of the nuclear staining of TTF1. We identified 2 distinct patterns of nuclear staining. For well-differentiated carcinomas lacking nodal metastases, extrathyroidal extension, or other adverse pathologic features, the staining was uniform and strong within the nucleus. Under circumstances with adverse

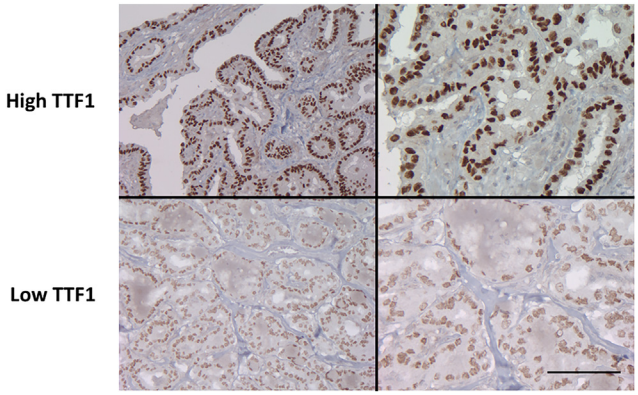

Fig. 1 Representative sections representing immunohistochemical staining of TTF1 in papillary thyroid carcinomas, scale bar indicates 50 μm.

pathology including either, or both, extrathyroidal extension and lymph node metastases, we observe a diminished staining intensity within the nucleus and in some cases only minimal nuclear membrane staining of TTF1. Overall, approximately 73% of specimens had strong staining that was consistent throughout the nucleus. Approximately 27% of all cases exhibited low levels of nuclear TTF1 which was diminished or limited almost exclusively to the nuclear membrane. Variations in the histologic description between classic papillary thyroid carcinomas and follicular variants did not account for changes in TTF1 staining ($P = .12$).

3.2. TTF1 and adverse pathologic features

The demographic variables for the patient groups defined by TTF1 staining patterns, including breakdown by stage at presentation, are shown in Tables 1 and 2. Overall, procedures completed for recurrent disease were more common in tumors exhibiting lower levels of nuclear TTF1. Patient age, sex, and follow-up timing were otherwise similar for both groups. All of the tumors were characterized for the presence of the *BRAF* mutation (59% overall), *ALK* mutations (2%), and adverse pathologic features (Table 2). On univariate analysis, low levels of nuclear TTF1 were associated with extrathyroidal extension ($P < .0001$), nodal metastases ($P < .0001$), and more advanced disease stage ($P = .0054$). Vascular invasion ($P < .0001$) was also linked to a paucity of nuclear TTF1 stain-

Table 1 Patient demographics and nuclear TTF1 staining intensity

Pathological characteristics	Cases (%) normal TTF1	Cases (%) low TTF1	*P*
No. of patients	350	135	
Female sex	252 (72%)	99 (73%)	.821
Duration of follow-up	58.4 ± 65.2	57.2 ± 63.6	.855
Age at first operation	47.3 ± 16.2	48.7 ± 16.6	.397
Procedures for recurrence	22 (6.3%)	20 (14.8%)	.006

Table 2 Nuclear TTF1 status and adverse pathologic features

Pathological characteristics	Cases (%) normal TTF1	Cases (%) low TTF1	*P*
Tumor size (mm)	21.02 ± 16.6	22.27 ± 16.4	.456
Extrathyroidal extension	98 (28%)	67 (50%)	<.0001
Vascular invasion	73 (21%)	58 (43%)	<.0001
Nodal metastases	105 (30%)	77 (57%)	<.0001
BRAFV600E positive	210 (60%)	76 (56%)	.472
Multifocal	148 (42.5%)	50 (37.6%)	.217
TNM stage	n = 350	n = 135	.0054
I	297 (84.8%)	101 (74.8%)	
II	44 (12.5%)	21 (15.5%)	
III	5 (1.4%)	6 (4.4%)	
IV	4 (1.1%)	7 (5.2%)	

Abbreviation: TNM, tumor-node-metastasis.

ing but not multifocality ($P = .217$). Specimens lacking any adverse pathological features or lymph node metastases (n = 203) typically exhibited almost uniformly strong nuclear TTF1 (84%). The presence or absence of the *BRAF* mutation ($P = .472$) did not influence the nuclear distribution of TTF1. Of the cases with ALK gene rearrangements (n = 7), none exhibited low levels of nuclear TTF1. In our multivariate analysis including pathological features, age, and sex, we observed that low levels of nuclear TTF1 were significantly correlated with the presence of nodal metastases ($P = .005$) and vascular invasion ($P = .019$).

3.3. TTF1, radioactive iodine activity, and disease recurrence

Disease recurrence was influenced by nuclear TTF1 levels as shown in Fig. 2. The increased rate of recurrence was similar across the timeline examined and did not favor early or

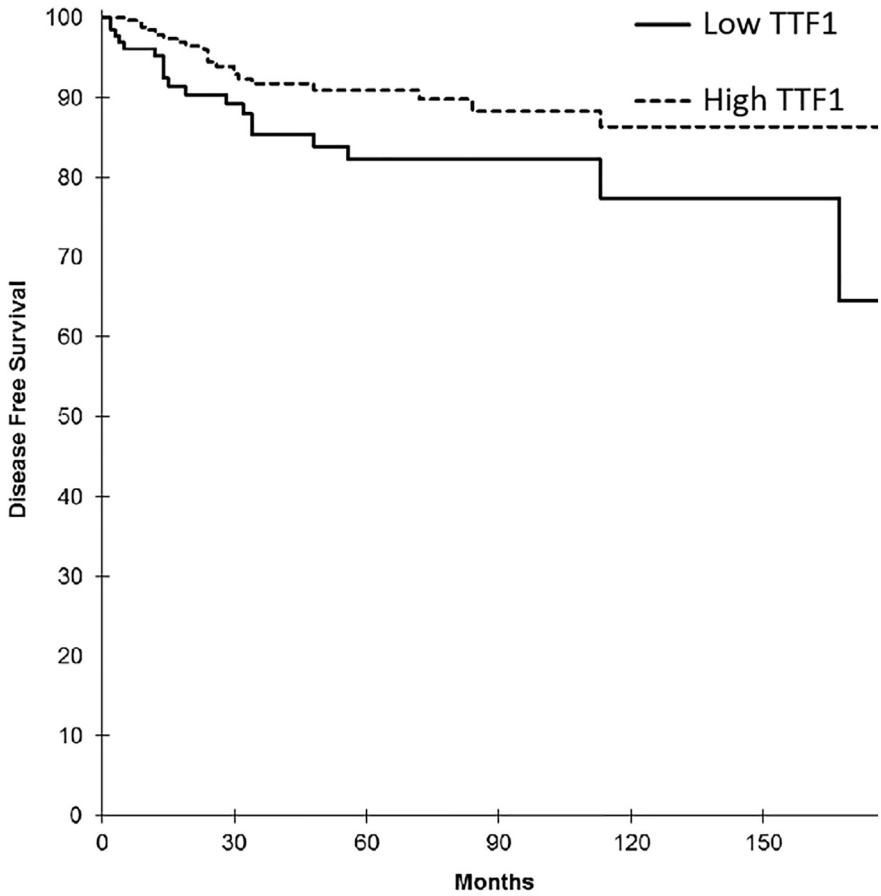

Fig. 2 Disease-free survival based on intensity of TTF1 staining in papillary thyroid cancer specimens.

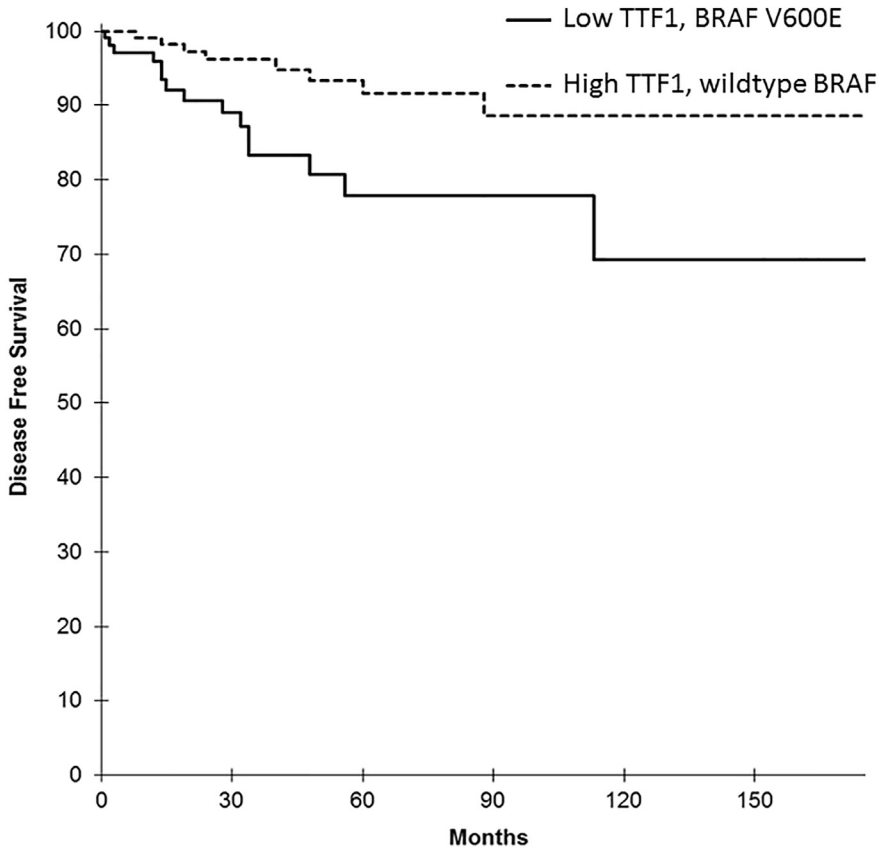

Fig 3 Disease-free survival based on intensity of TTF1 staining and the presence of the V600E *BRAF* mutation in papillary thyroid cancer specimens.

later disease. Interestingly, when patients with high levels of TTF1 staining and lacking the *BRAF* mutation were compared to patients with *BRAF* mutation and low nuclear TTF1 levels, we did observe a more dramatic difference in disease-specific survival (Fig. 3). Moreover, patient tumors positive for V600E *BRAF* mutation exhibiting low TTF1 levels had a higher T-stage (2.19 ± 1.02 versus 1.77 ± 0.89, $P = .0005$) and tended to be older (49.9 ± 16.3 versus 45.9 ± 16.3 years, $P = .054$). Radioactive iodine was also applied at higher activities in these patients compared to tumors with normal TTF1 and wild-type BRAF (5.86 ± 3.79 versus 4.44 ± 3.54 MBq, $P = .0028$).

4. Discussion

Differentiation of thyroid follicular cells depends on the nuclear localization of TTF1 to drive the functionality of thyroglobulin synthesis and iodide transport. We demonstrate a relationship between low nuclear TTF1 staining and the presence of aggressive thyroid pathologic features, notably vascular invasion, extrathyroidal extension, and lymph node metastases. Clear and consistent staining of the nucleus with TTF1 is almost uniformly linked to benign thyroid neoplasms and well-differentiated carcinomas exhibiting low metastatic potential. Disease recurrence favors low TTF1 levels, and this would be expected based on the association with adverse pathologic features. *BRAF* mutations are not linked to TTF1 expression and thus could not account for the changes in clinical behavior. However, the coexistence of poor TTF1 staining and mutated *BRAF* was predictive of further decreases in disease-specific survival. This may account for, in part, varying conclusions in the literature regarding patient outcomes and solitary assessment of the *BRAF* mutations [31-33] given potential variations in TTF1 as a confounder. Differences in nuclear TTF1 expression may provide another tool to examine the sum of genetic inputs that influence tumor behavior, and a model to understand dedifferentiation and poor outcomes in papillary thyroid carcinoma.

Previous studies indicate that TTF1 is lost with poorly differentiated and anaplastic thyroid carcinomas but that most tumors will exhibit some level of protein expression. The use of TTF1 as part of assessments in differentiation of thyroid carcinomas is relatively limited. One of the first studies to indicate the potential importance of nuclear staining of TTF1 was that of Zhang et al in 2006 [16]. In this study, more differentiated cancers exhibited higher levels of nuclear TTF1 staining, whereas Pax8 nuclear levels changed minimally. A second study examined TTF1 in normal and neoplastic tissue to reveal

strong staining within the nucleus as expected in benign and well-differentiated neoplasms [17]. However, in undifferentiated tumors, nuclear positivity of TTF1 by immunohistochemical assessment and in situ hybridization was weak or nonexistent. Bejarano et al (2000) noted that most cases of papillary thyroid carcinomas exhibited TTF1, but Hurthle cell variants were much more likely to have low levels of TTF1 [34]. Given the challenges in quantification in staining and the variations in sample selection, comparisons within this small number of case series are difficult. Consequently, most previous immunohistochemical studies over the past 2 decades have focused on the role of TTF1 as a diagnostic marker of thyroid or lung cancers but not as a prognostic tool in patients with thyroid disease [35].

We believe that there may be a spectrum of TTF1 functionality in thyroid carcinomas that is important to probe, but the regulation of differentiation in patient tumors remains unclear. Reports on *BRAF* and *TERT* mutations indicate varying prevalence and associations to adverse clinicopathological features [33,36,37]. Coexistent mutations do appear to drive adverse outcomes, but the associations are complex, and known clinical risk factors such as lymph node metastases are not always strongly linked to these mutations. Genomic studies examining RAS, BRAF, TP53, and other genetic markers have also failed to demonstrate any drivers regulating the transcription factors TTF1 and Pax8 [38]. The importance of continued efforts to understand TTF1 regulation however is reinforced by clear evidence for regulation of iodide transport and thyroglobulin production by TTF1 [23,39-42]. It appears that the regulation of TTF1 expression and function is posttranslational in most nonanaplastic thyroid carcinomas [17,43] and that a regulatory scheme for TTF1 may be related to phosphorylation and the balance between nuclear and cytoplasmic localization [27,44]. This could account for the observed variations in immunohistochemical assessments of TTF1 and the complexity of the genomic pathways that are required to define differentiation in thyroid cancer.

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