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Aberrant Expression of Thymosin Beta-4 Correlates With Advanced Disease and BRAF V600E Mutation in Thyroid Cancer

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Summary
Thymosin beta-4 (TMSB4X) was recently identified as a differentially expressed gene between malignant and non-malignant thyroid cells via single-cell RNA sequencing. In the present study, we aimed to study the immunostaining pattern of TMSB4X in benign and malignant thyroid neoplasms. Immunohistochemical analysis revealed that normal thyroid tissue or benign thyroid disorders exhibited undetectable immunoreactivity against TMSB4X except for positive staining of inflammatory infiltrates and stromal cells associated with autoimmune thyroid disease. By contrast, overexpression of TMSB4X was observed in a variety of thyroid malignancies, including papillary, follicular, poorly differentiated, and undifferentiated thyroid cancer. Among 141 patients with differentiated thyroid cancer, higher TMSB4X expression was associated with papillary tumor type, extrathyroidal extension, lymph node metastasis, and BRAF V600E mutation. The results were consistent with those from the public transcriptomic datasets. In summary, TMSB4X expression was aberrantly increased in various types of thyroid cancer, and higher TMSB4X expression was correlated with advanced disease characteristics. Thymosin beta-4 may be a novel downstream effector of the BRAF V600E mutation. (J Histochem Cytochem 70: 707–716, 2022)

Keywords
immunohistochemistry, thymosin beta-4, thyroid cancer, TMSB4X

Introduction

In general, thyroid cancer can be successfully treated with surgery, radioactive iodine, and thyrotropin suppression. As the issue of overdiagnosis and overtreatment of small microcarcinomas is recognized, overall incidence rates have declined, but incidence-based mortality rates continue to increase.[1] Diseases with poor prognosis, albeit uncommon, include aerodigestive tract invasion, distant metastasis, and radioiodine refractoriness. There are still unmet needs requiring refined risk stratification and more efficient therapeutics for advanced diseases. Substantial improvements in the understanding of the oncogenic pathways in thyroid cancer have led to innovative precision medications.[2] However, currently approved agents remain limited by their efficacy and toxicity profiles. Continuing efforts have been made to search for potential prognostic or predictive biomarkers to tailor personalized treatment strategies.

Single-cell RNA sequencing has been used to explore the heterogeneity and complexity of cell populations, evolutionary trajectories, and gene regulatory networks. Recently, a single-cell transcriptomic analysis of papillary thyroid cancer (PTC) reported that TMSB4X

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was significantly upregulated in malignant cells compared with non-malignant cells, and TMSB4X expression was increased in metastatic cells compared with primary cancer cells, suggesting that TMSB4X may be involved in thyroid cancer initiation and progression.[3] Thymosin beta-4, the gene product of TMSB4X located on the X chromosome, plays a pivotal role in a variety of physiological and pathological processes, particularly inflammation and angiogenesis.[4] Although differential expression patterns of thymosin beta-4 have been reported previously in other tumor types such as colorectal cancer and gastrointestinal stromal tumors,[5,6] the protein expression of TMSB4X in thyroid neoplasms has not been investigated.

In the current study, we aimed to study the immunostaining pattern of TMSB4X in benign and malignant thyroid samples and evaluate the relationship between TMSB4X expression and clinicopathological features in differentiated thyroid cancer.

Materials and Methods

Patient Population

Patients who underwent definitive surgery to treat thyroid cancer from 2001 to 2012 were randomly selected for tissue microarray construction.[7] The tumor area and paired normal-appearing adjacent thyroid tissue were manually reviewed by an experienced pathologist. Tumor–node–metastasis staging was based on the seventh edition of the American Joint Committee on Cancer staging system. Additional samples from the surgery for benign thyroid disorders were included for comparison. The institutional review board of MacKay Memorial Hospital granted approval for this study.

Immunohistochemical Analysis

Immunohistochemistry for TMSB4X protein was performed on 5-μm sections of formalin-fixed paraffin-embedded tissue microarray slides, and the procedure of immunodetection of TMSB4X was conducted as reported previously.[8] After deparaffinization and rehydration, antigen retrieval was performed in Tris-EDTA pH 9.0 in a microwave oven for 15 min. Following blocking, sections were incubated with a rabbit polyclonal anti-TMSB4X antibody (orb523491; Biorbyt, Cambridge, UK; dilution, 1:50) for 16 hr at 4C. After rinsing, a horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody (DAKO EnVision Systems; Agilent Technologies, Santa Clara, CA) was applied for 30 min. Diaminobenzidine and hematoxylin were used for the chromogenic reaction and counterstaining, respectively. Negative control was prepared using phosphate-buffered saline instead of the primary antibody, and a tumor sample from melanoma was used as a positive control.[9] In all cases, appropriate positive and negative controls were used.

Evaluation of Immunohistochemical Staining

Section images were acquired using the TissueFAXS platform (TissueGnostics; Vienna, Austria). All the slides were examined independently by two investigators in concordance. Tumor cells were identified by orientation, cell size, and nuclear configuration. For semiquantitative evaluation, the widely accepted histological score (*H*-score) method was used.[10] Staining intensity of the principal expression pattern was scored on a 4-point scale (0: negative, 1: weak, 2: moderate, 3: intense) along with the percentage of tumor cells (0–100%) that exhibited the staining pattern. The product of the staining intensity and the percentage of positive cells were calculated to generate an *H*-score for each tumor.

Detection of the BRAF V600E Mutation

DNA were extracted from tissue blocks, and Sanger sequencing was performed to detect the BRAF c.1799T>A (p.Val600Glu) mutation. The sensitivity of BRAF V600E detection using Sanger sequencing is approximately 15% mutant allele frequency.[11]

Public Transcriptomic Datasets

For validation purposes, datasets were downloaded from public repositories and analyzed. The GSE29265 dataset contained gene expression profiling of 20 non-tumor controls, 20 PTCs, and 9 anaplastic thyroid cancers using the Affymetrix Human Genome U133 Plus 2.0 Array.[12] The GSE54958 dataset contained expression profiles of 7 normal thyroids, 7 follicular adenomas, 11 BRAF-wild-type PTCs, and 14 BRAF-mutant PTCs using the Affymetrix Human Gene 1.0 ST Array.[13] The GSE60542 dataset had genome-wide mRNA profiles from 31 patients with PTC and matched non-cancerous thyroid and lymph node samples using the Affymetrix Human Genome U133 Plus 2.0 Array.[14] The Cancer Genome Atlas (TCGA) thyroid cancer (THCA) dataset was obtained from the Genomic Data Commons Data Portal of the National Cancer Institute.[15] The ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumors using Expression data) algorithm was used to appraise the extent of infiltrating stromal and immune cells in TCGA samples.[16]

Statistical Analysis

Differences in clinicopathological features across different TMSB4X expression groups were evaluated using the Cochran–Armitage trend test and the Jonckheere–Terpstra test. Comparisons of *H*-scores and TMSB4X expression between and among groups were made using the Mann–Whitney *U* test, Wilcoxon signed-rank test, Kruskal–Wallis test, or Jonckheere–Terpstra test, as appropriate. To evaluate the relationship between continuous variables, Spearman's rank correlation was used.[17] All hypothesis tests were two-sided, and statistical significance was set at $p<0.05$.

Results

Expression of TMSB4X in Thyroid Tissue and Tumors

Immunohistochemical staining showed that normal thyroid tissue ($n=141$), nodular goiter ($n=10$), or follicular adenomas ($n=10$) exhibited undetectable or barely detectable immunoreactivity against TMSB4X (Fig. 1). In Graves' disease ($n=10$) or lymphocytic thyroiditis ($n=10$), TMSB4X staining was predominantly present in inflammatory infiltrates and stromal cells. Positive TMSB4X immunostaining was seen in mononuclear and polymorphonuclear leukocytes but not erythrocytes. Thyroid follicular epithelial cells had indiscernible or sparse intracellular TMSB4X expression in autoimmune thyroid disorders.

By contrast, overexpression of TMSB4X was observed in different types of thyroid malignancies, including papillary ($n=124$), follicular ($n=17$), poorly differentiated ($n=7$), and undifferentiated ($n=2$) thyroid cancer. The expression was characterized primarily by a diffuse homogeneous staining of the entire cytoplasm (Fig. 2). A spot-like granular pattern with perinuclear accentuation was also common. In a few cancer cells, even nuclear staining was evident. Overall, TMSB4X immunostaining was aberrantly upregulated in transformed thyroid cancer cells at significantly higher levels than those in normal or benign thyroid cells.

Correlation Between TMSB4X Expression and Clinicopathological Factors

To establish a clinicopathological correlation of TMSB4X expression in thyroid cancer, *H*-scores in 141 patients with differentiated thyroid cancer were further analyzed. The median (interquartile range) *H*-score was 170 (102.5–255) for PTC and 50 (30–130) for follicular thyroid cancer. TMSB4X *H*-scores were positively associated with extrathyroidal extension, lymph node metastasis, and tumor–node–metastasis staging (Fig. 3). Information on BRAF mutation status was available in 136 patients, and 87 (64%) had the BRAF V600E mutation. Thyroid cancer with the BRAF V600E mutation had significantly higher TMSB4X *H*-scores than those with wild-type BRAF ($p<0.001$).

We categorized TMSB4X *H*-scores into tertiles and divided the patient cohort into low (*H*-score, 0–100), intermediate (*H*-score, 101–200), and high (*H*-score, 201–300) expression groups. As shown in Table 1, higher TMSB4X expression was associated with papillary tumor type ($p=0.001$) and older patient age at diagnosis ($p=0.024$). Moreover, extrathyroidal extension ($p=0.008$), lymph node metastasis ($p=0.024$), and BRAF V600E mutation ($p<0.001$) were positively associated with TMSB4X expression. Together, these findings suggest that TMSB4X expression correlates with advanced disease in differentiated thyroid cancer.

External Validation of TMSB4X Expression in Thyroid Cancer

We performed analyses on public datasets as a means of external validation. The GSE29265 dataset showed that both papillary and anaplastic thyroid cancer had higher TMSB4X expression than normal thyroid tissue, and the GSE54958 dataset indicated that PTC had higher expression than normal thyroid tissue and follicular adenoma (Fig. 4A and B). PTC with a BRAF mutation tended to have higher TMSB4X expression than wild-type cancer, but the difference did not reach statistical significance in the GSE54958 dataset. Interestingly, the GSE60542 dataset indicated that TMSB4X expression was progressively increasing from non-cancerous thyroid tissue to primary tumors, and further to metastatic lymph nodes (Fig. 4C). Analysis of 59 matched normal-tumor pairs in the TCGA dataset also confirmed that tumors had significantly higher RNA expression levels than their normal counterparts (Wilcoxon signed-rank test, $p<0.001$).

Consistent with immunohistochemical results, RNA sequencing (RNA-seq) data from 501 THCA samples in the TCGA dataset revealed that tumors with extrathyroidal extension (Fig. 4D) or lymph node metastasis ($p<0.001$) exhibited higher TMSB4X expression. The follicular fraction, a histopathological measure of the fraction of tumor cells with a preserved follicular architecture, was negatively correlated with TMSB4X expression (Spearman's rho = –0.469, $p<0.001$). Accordingly, there was a highly negative correlation

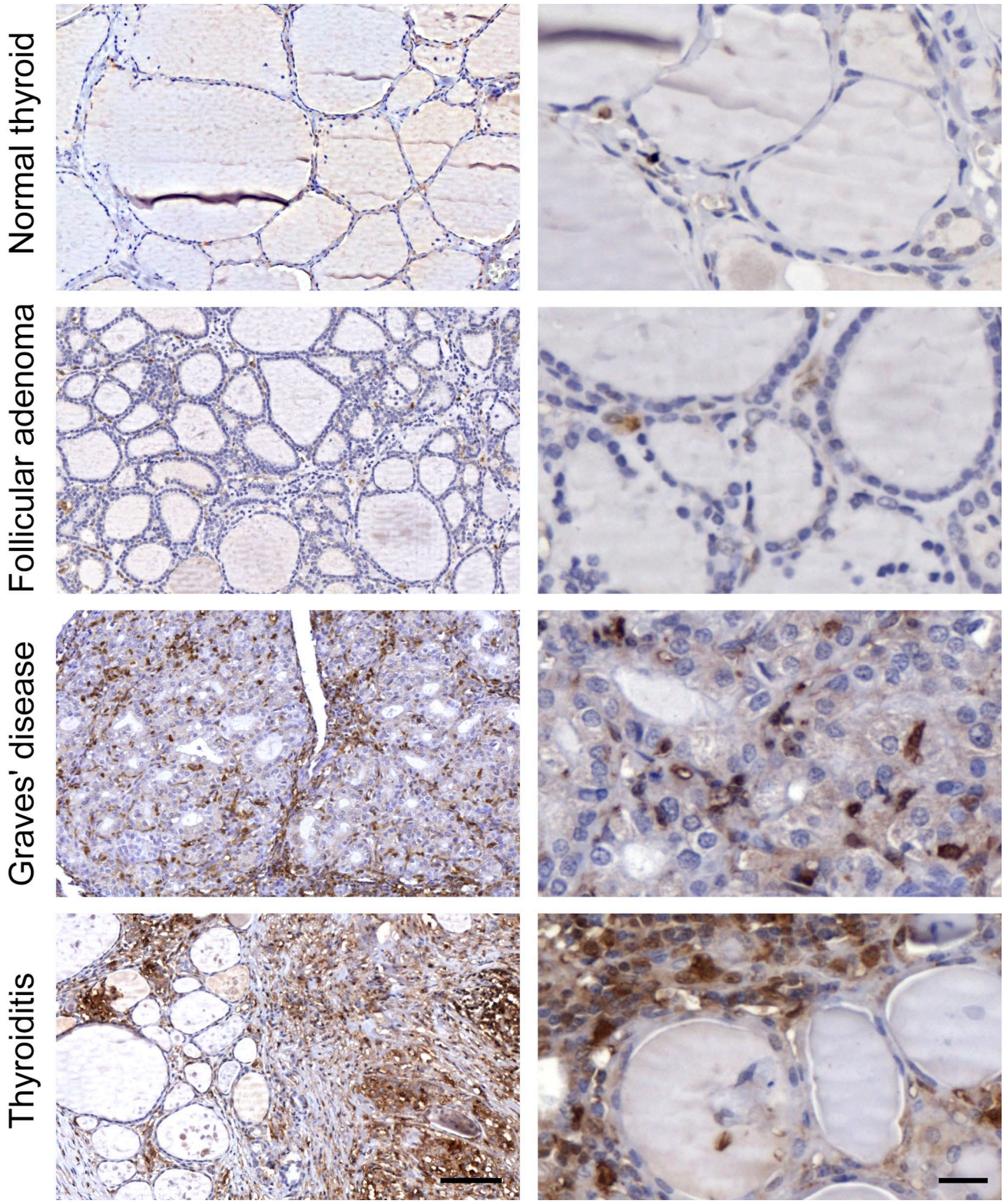

Figure 1. Expression of thymosin beta-4 (TMSB4X) in normal thyroid and benign thyroid disorders. Representative immunohistochemical microphotographs are shown. Scale bars: left panel 100 μm, right panel 20 μm.

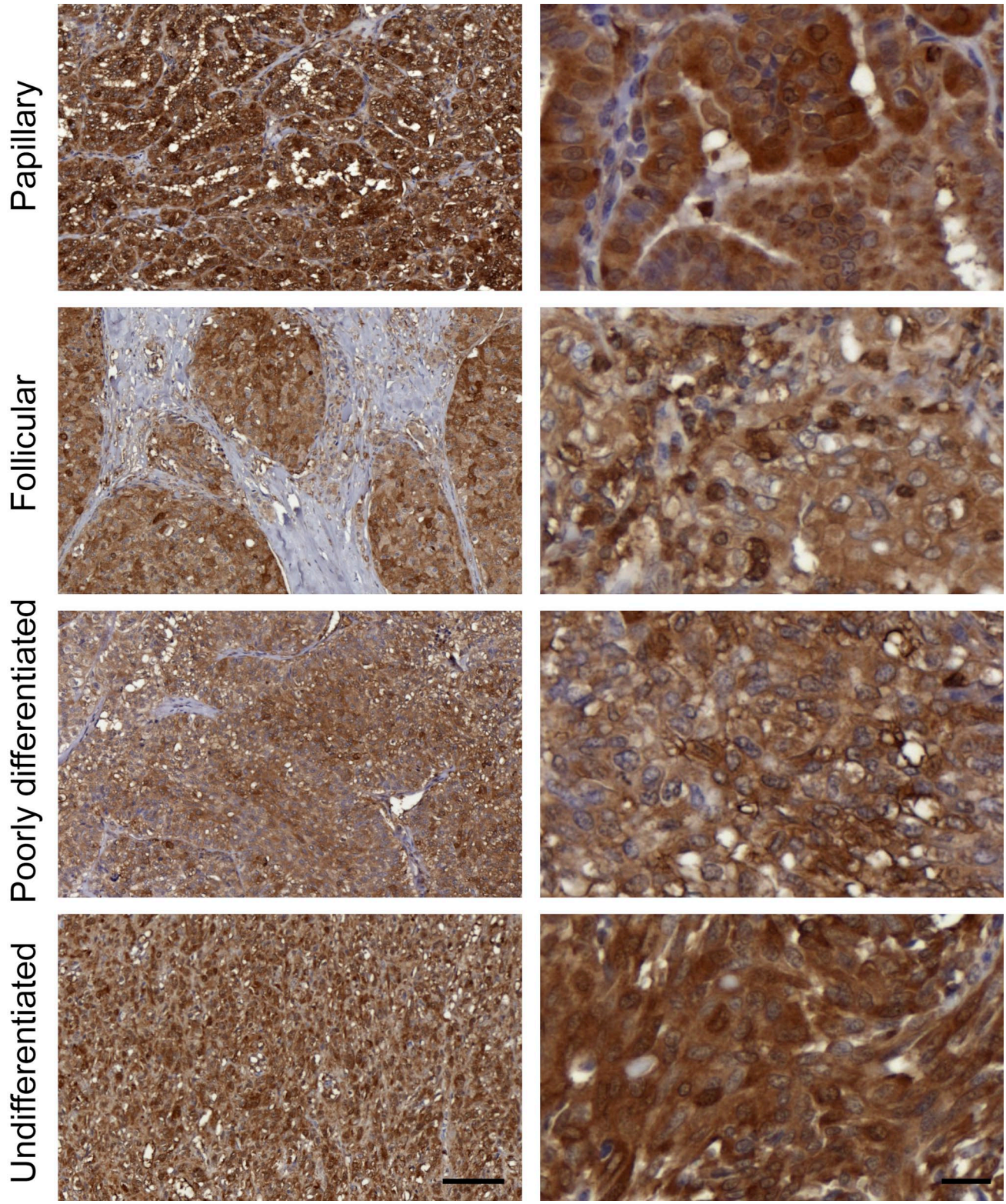

Figure 2. Expression of thymosin beta-4 (TMSB4X) in malignant thyroid tumors. Representative immunohistochemical microphotographs are shown. Scale bars: left panel 100 μm, right panel 20 μm.

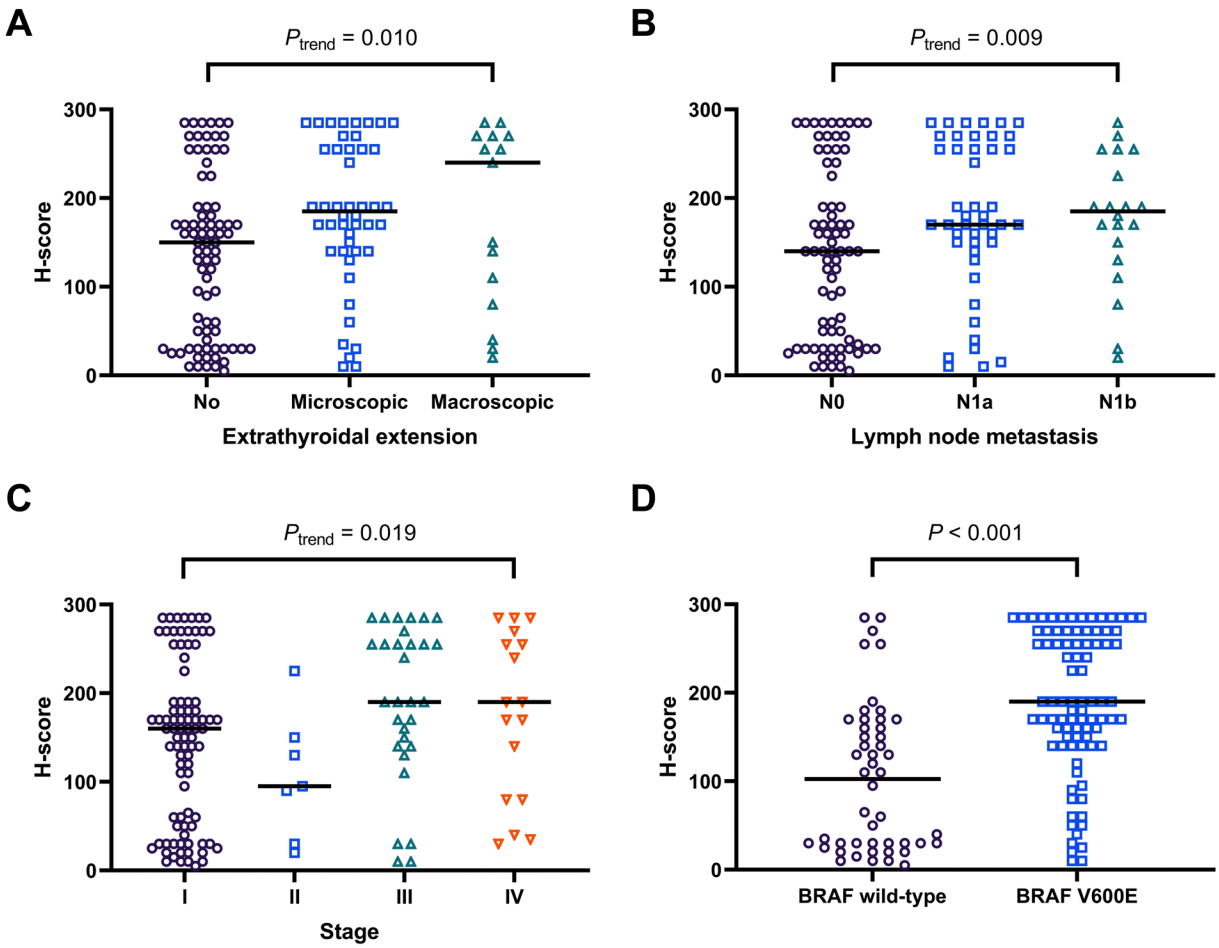

Figure 3. Clinicopathological correlation with immunohistochemical expression of thymosin beta-4 (TMSB4X) in 141 patients with differentiated thyroid cancer. Scatter plots with medians are shown for immunohistochemical *H*-scores vs (A) extrathyroidal extension, (B) lymph node metastasis, (C) tumor–node–metastasis stage, and (D) BRAF V600E mutation status. *P* values were calculated using the Mann–Whitney *U* test or Jonckheere–Terpstra test.

(Spearman's rho = –0.560, $p<0.001$; Fig. 4E) between TMSB4X expression and the thyroid differentiation score, a measure of gene expression for 16 thyroid function genes developed by the initial global TCGA THCA analysis.[15]

Given our observed positive TMSB4X immunostaining of inflammatory infiltrates and stromal cells in autoimmune thyroid disorders, we examined the relationship between TMSB4X expression and stromal and immune cell components. Of interest, TMSB4X expression in the TCGA dataset had highly positive correlations with the ESTIMATE-derived stromal score (Spearman's rho = 0.442, $p<0.001$) and immune score (Spearman's rho = 0.606, $p<0.001$; Fig. 4F). These results suggest that RNA expression levels from microarray or RNA-seq experiments represent a combined expression estimate of tumor cells and stromal and immune cells. Nonetheless, results from transcriptome analyses were in good agreement with the present immunohistochemical study, substantiating that thymosin beta-4 is a marker for aggressive characteristics in thyroid cancer.

Discussion

In this study, we for the first time investigated the protein expression of TMSB4X in benign and malignant thyroid neoplasms. We found that TMSB4X was aberrantly overexpressed in the vast majority of thyroid cancers, irrespective of their differentiation status. Using serial analysis of gene expression (SAGE), TMSB4X was previously identified to be differentially expressed between follicular thyroid cancer and follicular adenoma.[18] A subsequent study using cDNA arrays showed that PTC had higher TMSB4X expression than follicular thyroid cancer.[19] Our findings are

Table 1. Clinicopathological Correlation With Expression of TMSB4X in 141 Patients With Differentiated Thyroid Cancer.

	TMSB4X expression			
	Low (*n*=41)	Intermediate (*n*=57)	High (*n*=43)	*P* value
Type				0.001
Papillary	31 (76%)	50 (88%)	43 (100%)	
Follicular	10 (24%)	7 (12%)	0 (0%)	
Sex				0.166
Female	34 (83%)	49 (86%)	40 (93%)	
Male	7 (17%)	8 (14%)	3 (7%)	
Age (years)	38 (31–53)	44 (34–54)	47 (39–58)	0.024
Hashimoto thyroiditis				0.455
Absent	40 (98%)	50 (88%)	40 (93%)	
Present	1 (2%)	7 (12%)	3 (7%)	
Tumor size (cm)				0.057
0–2 cm	13 (32%)	22 (39%)	21 (49%)	
2–4 cm	22 (54%)	30 (53%)	20 (47%)	
>4 cm	6 (15%)	5 (9%)	2 (5%)	
Extrathyroidal extension				0.008
No	30 (73%)	31 (54%)	19 (44%)	
Microscopic	7 (17%)	23 (40%)	16 (37%)	
Macroscopic	4 (10%)	3 (5%)	8 (19%)	
Lymphovascular invasion				0.895
Absent	31 (76%)	44 (77%)	32 (74%)	
Present	10 (24%)	13 (23%)	11 (26%)	
Lymph node metastasis				0.024
N0	30 (73%)	26 (46%)	20 (47%)	
N1a	8 (20%)	20 (35%)	17 (40%)	
N1b	3 (7%)	11 (19%)	6 (14%)	
TNM stage				0.050
I	28 (68%)	38 (67%)	21 (49%)	
II	4 (10%)	2 (4%)	1 (2%)	
III	4 (10%)	12 (21%)	14 (33%)	
IV	5 (12%)	5 (9%)	7 (16%)	
BRAF V600E mutation[a]				<0.001
Absent	24 (63%)	19 (35%)	5 (12%)	
Present	14 (37%)	36 (65%)	37 (88%)	

Values are presented as frequency (percentage) or median (interquartile range).
Abbreviation: TNM, tumor–node–metastasis; TMSB4X, thymosin beta-4.
[a]Data missing from six patients.

in agreement with these observations. Normal or benign thyroid follicular epithelium was essentially negative for TMSB4X immunostaining, whereas protein expression levels of TMSB4X were upregulated in the transformed malignant cells, particularly the papillary tumor type.

Our clinicopathological correlation revealed that higher TMSB4X expression was associated with several aggressive features of carcinoma, such as extrathyroidal extension and lymph node metastasis. The results of the immunohistochemical study were consistent with the RNA-seq analysis of the TCGA dataset. In breast cancer, TMSB4X overexpression was significantly correlated with lymph node metastasis.[20] In colorectal cancer, the strongest expression for TMSB4X was detected in the invasion front with features of epithelial–mesenchymal transition (EMT).[21] Experimental evidence suggests that TMSB4X is linked to multiple EMT-related pathways, such as integrin-linked kinase, Wnt/β-catenin signaling, and transforming growth factor beta signaling pathways.[22–24] Interestingly, the natural tetrapeptide *N*-acetyl-Ser-Asp-Lys-Pro, a degradation product of thymosin beta-4, was found to be increased in the intratumoral blood but not in the

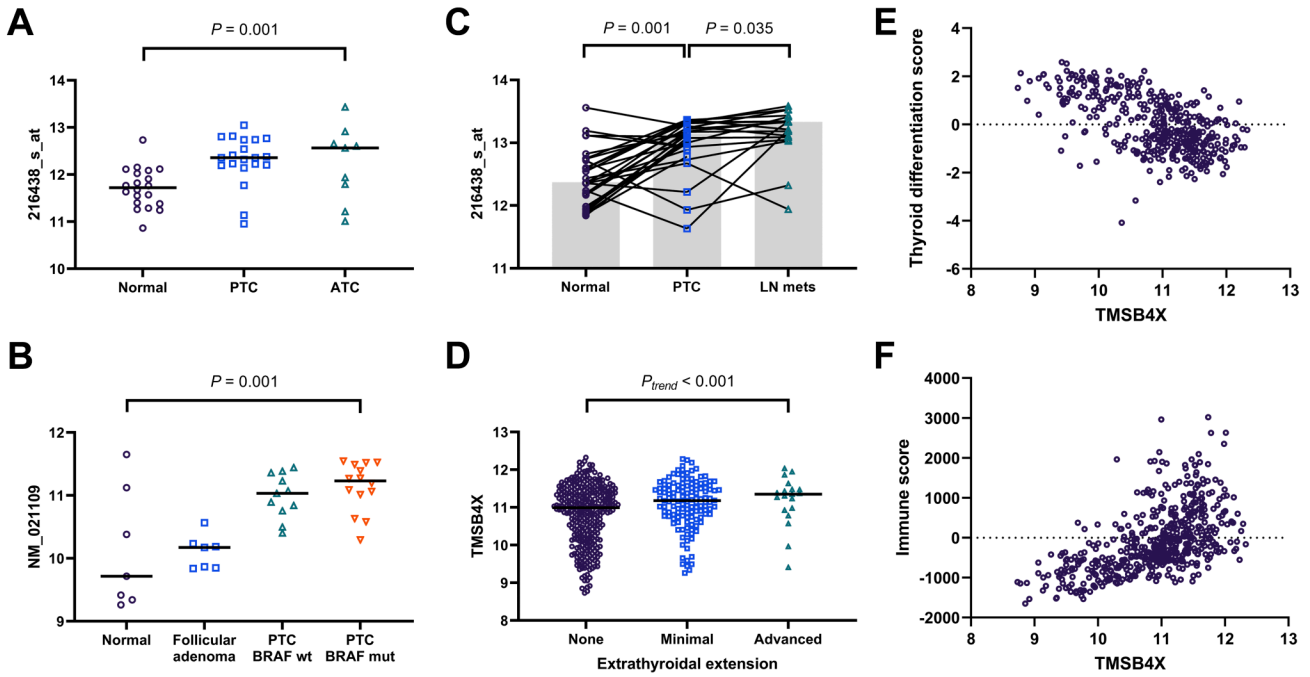

Figure 4. External validation of thymosin beta-4 (TMSB4X) expression in public transcriptomic datasets of thyroid cancer. Horizontal lines and bars in scatter plots represent the medians. (A) The GSE29265 dataset. ATC, anaplastic thyroid cancer; PTC, papillary thyroid cancer. *P* value was calculated using the Kruskal–Wallis test. (B) The GSE54958 dataset. mut, V600E mutation; wt, wild-type. *P* value was calculated using the Kruskal–Wallis test. (C) The GSE60542 dataset. LN mets, nodal metastases. *P* values were calculated using the Wilcoxon signed-rank test. (D)–(F) The Cancer Genome Atlas (TCGA) thyroid cancer (THCA) dataset. *P* for trend was calculated using the Jonckheere–Terpstra test.

peripheral blood of patients with PTC.[25] These results suggest that TMSB4X promotes EMT in an autocrine or paracrine manner but not an endocrine manner.

We found that TMSB4X immunostaining was present in inflammatory and stromal cells associated with autoimmune thyroid disease. This is consistent with previous observations that stromal cells showed weak to moderate TMSB4X staining, whereas leukocytes showed medium to strong TMSB4X staining.[26] An unexpected finding in the current study is that TMSB4X immunoreactivity in thyroid cancer epithelial cells appeared to increase in parallel with patient age. It should be emphasized that age is an important prognostic factor in differentiated thyroid cancer. Older age is independently associated with extrathyroidal extension and lymphovascular invasion.[27,28] The association between patient age and TMSB4X expression observed in this study may be biased by the fact that advanced disease was more frequent in elderly patients than in younger patients. Recently, we demonstrated that alterations in immune and inflammatory responses were characterized in the thyroid transcriptome during aging.[29] The role of TMSB4X in reciprocal interactions between thyroid cancer epithelium and microenvironment could be a promising avenue for future research.

The BRAF V600E mutation is the most common somatic mutation in thyroid cancer. Of note, we uncovered a close relationship between the BRAF V600E mutation and TMSB4X expression. This may partially explain the observation that PTC had higher TMSB4X expression compared with follicular thyroid cancer. Consistent with our results, the Johns Hopkins group reported that follicular fraction, a proxy indicator for assessing thyroid differentiation, was lower in BRAF-like tumors than in RAS-like tumors.[30] It is worth noting that thymosin beta-4 can stimulate angiogenesis and accelerate wound heading.[31] In malignant tumors, TMSB4X expression frequently correlates with the expression of vascular endothelial growth factor or hypoxia-inducible factors.[6,20] The BRAF V600E mutation was shown to increase angiogenesis in thyroid cancer.[32] We postulated that TMSB4X is probably another link between the BRAF V600E mutation and the angiogenic switch.

Although the TMSB4X gene is located on the X chromosome, we did not detect differences in TMSB4X expression between the sexes. Consistently, quantification of TMSB4X transcripts did not differ between males and females in the GSE60542 dataset (p=0.924) and the TCGA dataset (p=0.522). In addition, gender did not influence the expression levels of TMSB4X in

head and neck cancer.[33] A plausible explanation is that lyonization (X-inactivation) balances the gene expression of TMSB4X.

In summary, we disclosed that TMSB4X immunohistochemical expression was aberrantly increased in various types of thyroid cancer. In differentiated thyroid cancer, higher TMSB4X expression was correlated with advanced disease characteristics such as extrathyroidal extension and lymph node metastasis. Furthermore, a close association between TMSB4X expression and the BRAF V600E mutation suggests that thymosin beta-4 may be a novel downstream effector of the BRAF V600E oncogene. In addition to diagnostic implications, TMSB4X expression in initial tumor samples is a potentially actionable prognostic indicator of thyroid cancer.

Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

C-YK and S-PC helped with the conception and design of the study; C-YK, J-YJ, W-CH, and S-PC with the acquisition and analysis of data; and C-YK and S-PC with the drafting of the manuscript and the figures. All authors read and approved the final manuscript.

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Ethics Approval

This study was approved by the institutional review board of MacKay Memorial Hospital (12MMHIS149).

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