Tumor immune microenvironment characteristics of papillary thyroid carcinoma are associated with histopathological aggressiveness and BRAF mutation status

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Abstract

Background: Papillary thyroid carcinoma (PTC) follows an indolent course; however, up to 30% of patients develop recurrent disease requiring further treatment. Profiling PTC immune complexity may provide new biomarkers for improved risk prediction.

Methods: Immune complexity profiles were quantitatively evaluated by multiplex immunohistochemistry (mIHC) in archived tissue sections from 39 patients with PTC, and were assessed for correlations with aggressive histopathological features based on the presence of lymphovascular invasion and/or extrathyroidal extension, and BRAF V600E mutational status.

Results: mIHC revealed two distinct immune clusters stratifying patients: a lymphoid-inflamed group (higher CD8+ T cells, reduced dendritic and mast cells) and a myeloid/hypo-inflamed group that correlated with aggressive pathological features. BRAF mutation was not associated with aggressive pathological features but did correlate with increased mast cell density.

Conclusions: Distinct immune microenvironments exist in PTC correlating with pathological aggressiveness. Immune-based biomarkers associated with possible tumor-immune interactions may be used for risk stratification.

KEYWORDS
biomarker, immune cell, multiplex immunohistochemistry, thyroid cancer, tumor microenvironment

Casey Means and Daniel R. Clayburgh contributed equally to this study.
1 | INTRODUCTION

Papillary thyroid cancer (PTC) is the most common type of endocrine malignancy, with an estimated 40 000 new cases in 2015.\textsuperscript{1} PTC has shown an increasing incidence over the past 35 years, while follicular, medullary, and anaplastic varieties of thyroid cancer have remained stable.\textsuperscript{1,2} Although PTC tends to have favorable 5-year survival rates of greater than 90%, this decreases to <60% when there is distant spread.\textsuperscript{3} Potential pathological aggressiveness of PTC may manifest as locoregional and lymphatic spread; 30% of patients present with clinically evident cervical nodal disease, and up to 80% of patients are known to possess microscopic nodal disease. Current therapies for PTC are not benign, with surgical therapy of primary tumor and regional disease followed by risk-based use of adjuvant radioactive iodine treatment or external beam radiation therapy. These therapies do not come without a cost, thus improving risk stratification for PTC remains a priority in order to avoid overtreatment of this disease.

Historically, it was thought that leukocytes in close proximity to tumors represented an effort by the host to eradicate malignant cells. However, it is now known that the interaction is much more complex, and there may be subsets of immune cells that actually promote growth or facilitate survival of neoplastic cells.\textsuperscript{3–5} Tumors employ numerous mechanisms preventing elimination by the immune system, including poorly immunogenic mutations, secretion of mediators that inactivate cytotoxic immune cells, loss of major histocompatibility antigen expression, and secretion of factors that promote angiogenesis, matrix remodeling, and recruitment of both pro-tumoric and anti-tumoric leukocytes.\textsuperscript{4,6} Adding to this complexity, the biochemical and cellular milieu of tumors variably activates immune cells, promoting either pro-tumor or antitumor behaviors based on cell-cell interactions and local soluble molecules correlating with the biologic behavior of the tumor immune microenvironment (TiME); however, the full complexity of the TiME has not been fully explored in PTC.

In order to more fully evaluate TiMEs of PTC, we developed multiplex immunohistochemistry (mIHC) methodology for comprehensive profiling of immune cell infiltrates, thus leading to improved understanding of tumor pathophysiology.\textsuperscript{7} While previous reports described the prognostic significance of lymphocytes in PTC,\textsuperscript{4,8–10} the present study reports on simultaneous evaluation of CD8\textsuperscript{+} T cells, helper T cells, regulatory T cells (T\textsubscript{REG}), B cells, natural killer (NK) cells, CD68\textsuperscript{+}CSF1R\textsuperscript{+} tumor-associated macrophages (TAMs), CD66\textsuperscript{+} granulocytes, mature dendritic cells, and mast cells in a cohort of PTCs. Results from this TiME assessment of formalin-fixed, paraffin-embedded (FFPE) tissue sections reveal immune comprehensive characteristics of aggressive PTC, contributing to disease pathogenesis that may improve risk stratification and prognosis, as well as identify new targets for therapy.

2 | METHODS

2.1 | Clinical samples

FFPE samples of previously resected PTC (N = 39) were obtained from the Oregon Health and Science University (OHSU) Knight Cancer Institute Biobrary. Patients underwent thyroidectomy between 2011 and 2012 at OHSU. Samples were divided into those with or without aggressive histopathological features identified by the presence of lymphovascular invasion and/or extrathyroidal extension including microscopic and gross invasion. All tumor samples were reviewed by a senior pathologist specializing in head and neck tumors (D.S.). FFPE blocks were cut into 5 \( \mu \text{m} \) sections by the OHSU Histology Shared Resource Core. Electronic health records were used to identify anti-Tg and anti-TPO status, along with patient demographics and tumor information. The study was approved by the Institutional Review Board (#9420) at OHSU, and written informed consent was obtained from all patients.

2.2 | Multiplex IHC

mIHC was performed as previously described.\textsuperscript{7} Briefly, FFPE tumor sections were subjected to sequential immunodetection with antibodies detecting immune cell lineages (Supporting information Tables S2 and S3). Following chromogen development of antibodies, slides were digitally scanned using an Aperio ScanScope AT at x20 magnification. Tissue sections were then stripped of antibody and chromogen followed by subsequent rounds of antibody staining and imaging. A complete list of antibodies and conditions used for staining is provided in Supporting Information Table S2. Antibodies and order of staining was optimized for use in thyroid tissue. Following staining and image acquisition, computational processing was performed as previously described.\textsuperscript{7} Digital images reflecting the antibody panel were co-registered and aligned (Figure 1A). A sequential gating strategy was then utilized to identify immune cell phenotypes based on positive and negative cell staining (Figure 1B and Supporting Information Table S1). Due to computational limitations which do not allow for the 11-marker biomarker composition of the entirety of a large surgical tissue specimen to be analyzed, the three highest density leukocyte regions in any given tissue section were identified by CD45-positivity, approximately 25 000 000 pixels\textsuperscript{2} or 6.25 mm\textsuperscript{2} each, and quantitatively analyzed (Figure 2) as compared to adjacent benign tissue selected from a single region of interest (ROI) of comparable size. Image cytometry analysis was performed and results were compared between intra-tumor and adjacent benign regions.
2.3 | BRAF mutational testing

All tumor samples were evaluated for BRAF V600E mutations. Qiagen DNA mini kits were utilized to extract genomic DNA from FFPE tissue sections. BRAF Exon 15 (V600) was amplified by PCR (35 cycles), utilizing the following primers: Forward primer GCTTGCTCTGATA GGAAAATGAGA; Reverse primer GCAGCATCTCA GGGCCAAA. PCR products were then sequenced using Sanger sequencing and analyzed for the T1799A mutation.

2.4 | Statistics

Chi-squared test, Kruskal-Wallis test, and Wilcoxon signed-rank test were used to determine statistically significant differences. P values were adjusted for multiple comparisons using Benjamini-Hochberg false discovery rate adjustments. Statistical calculations were performed by R software, version 3.2.3 (http://www.r-project.org/). An unsupervised hierarchical clustering was performed with Ward's minimum
3 | RESULTS

3.1 | Immune microenvironment of PTC exhibits intratumoral myeloid predominance

A total of 39 subjects with PTC were included in this study (Table 1). There were no significant differences between the pathologically aggressive and indolent cohorts with respect to age, sex, or pT classification. All patients were female. Average age at diagnosis was 34.5 and 37.6 years old, respectively.

In order to study the TiME in these samples, mIHC and image cytometry analysis was performed to quantitatively identify differences in the immune microenvironment between intra-tumor and adjacent nonmalignant compartments (Figures 1A,B and 2). While nonmalignant regions exhibit enriched lymphoid cell densities, for example, CD8\(^+\) T cells and helper T cells, as compared to intratumoral regions (Figure 3A,B), intratumoral microenvironments were enriched with myeloid cell populations that included TAMs, granulocytes, and mast cells, as compared to nonmalignant regions (Figure 3C). Interestingly, intratumoral regions exhibited increased percentages of T\(_{\text{REG}}\) cells (Figure 3C), which are known to suppress cytotoxic T cell immune responses.\(^{11}\) No statistically significant differences were observed in NK cells, B cells, or dendritic cells between the two compartments. We examined ratios of CD8\(^+\) T cell:TAM, and CD8\(^+\) T cell: T\(_{\text{REG}}\)\(^{12–14}\) and found significantly lower ratios in intratumoral areas as compared to adjacent benign (Figure 3D,E), indicating that intratumoral regions likely harbor cell-based mechanisms that suppress cytotoxic properties of CD8\(^+\) T cells as compared to surrounding adjacent nonmalignant tissue.

3.2 | Intratumoral myeloid-inflamed profiles correlate with pathological aggressiveness of PTC

In order to assess the relationship between TiME composition and tumor aggressiveness, cell densities of nine immune cell lineages were subjected to unsupervised hierarchical variance method (hclust from R, http://sekhon.berkeley.edu/stats/html/hclust.html). All P values <0.05 were considered statistically significant.

FIGURE 2 Region of interest (ROI) selection based on papillary thyroid cancer (PTC) leukocyte hot spot analysis. Overview of mapping analyses for CD45\(^+\) leukocyte cell densities is shown. Following generation of whole tissue-based pseudo-immunohistochemistry (IHC) images from hematoxylin and CD45-IHC images (middle top panel), heat maps of leukocyte cell density were generated based on quantification of CD45\(^+\) cells per area (middle bottom panel). Then, based on pathologist-identified malignant regions, the three highest CD45\(^+\) density regions within malignant regions were selected and exported as ROIs for downstream image analysis (left panels). The highest leukocyte density region in adjacent nonmalignant tissue was also extracted and similarly quantitatively evaluated (right panels). Magnification is shown and boxes show the area magnified [Color figure can be viewed at wileyonlinelibrary.com]
This resulted in two distinct immune subpopulations within the cohort (Figure 4A and Supporting Information Table S3); one cluster appeared to be relatively hypo-inflamed and myeloid predominant, exhibiting lower numbers of CD8+ T cells, and higher numbers of mast cells and dendritic cells (Figure 4B-D), whereas the second cluster was characteristically lymphoid inflamed. The hypo-inflamed myeloid predominant group encompassed the majority of samples with aggressive pathological features (Figure 4E) and also correlated with a lower ratio of CD8+ T cells to total T cells, CD8+ T cells to TREG cells, and CD8+ T cells to TAMs (Figure 4F). Together, these findings indicate that a relatively hypo-inflamed myeloid-pre-dominant TiME correlates with pathological aggressiveness of PTC.

### 3.3 | BRAF V600E mutation status is associated with differential immune profiles of PTC

Previous reports have revealed that known genetic mutations in PTC activate transcriptional pro-inflammatory programs\(^ {15-17}\); thus, we evaluated BRAF V600E mutational status, in order to evaluate genetic mutational status in comparison to differential immune complexity of PTC. Results from this analysis (Figure 4A) indicated that BRAF V600E mutation status did not correlate with either lymphoid or myeloid inflamed clusters or pathological aggressiveness (Figure 5A); however, the BRAF V600E mutation positive group did display a significantly increased mast cell density as compared with the BRAF wild-type group (Figure 5B), and also exhibited tendencies toward increased mast cell percentages as compared to CD8+ T cell percentages, possibly revealing an immunosuppressive relationship between the two cell types (Figure 5C).

### 4 | DISCUSSION

PTC poses a unique challenge to clinicians; although most cases are relatively indolent, a small subset exhibits aggressive behavior and entails significant morbidity. Improved risk stratification of patients is thus needed to more accurately tailor therapy to individual patients. One important factor that may be critical to PTC behavior is the poorly understood tumor-immune interface. Thus, in the present study, we utilized an innovative mIHC process and quantitative bioinformatics to comprehensively assess immune cell complexity in PTC. This analysis revealed significant differences in intratumoral vs adjacent tissue immune microenvironments, and revealed that tumors with aggressive pathological features exhibit a predominantly myeloid TiME composition as compared to nonaggressive tumors characterized by a more lymphoid-inflamed phenotype.

Previous analysis of immune cell composition of in thyroid carcinoma (and other cancers) has been limited by the tools available, in particular flow cytometry and traditional IHC. Although flow cytometry can provide valuable information regarding the relative numbers of various cell populations, there is no spatial information regarding distribution of cells, and it requires fresh tissue rather than readily available FFPE specimens. Alternatively, traditional IHC is...
FIGURE 3 Papillary thyroid cancers (PTCs) exhibit intratumoral myeloid predominance. A, A representative case shows intratumoral predominance of myeloid lineages. Top panels show hematoxylin images to indicate regions with intratumoral PTC and adjacent benign regions discerned by hashed lines. Middle panels show dot plots to indicate differential distribution of total CD45+ cells, CD8+ T cells, regulatory T cells (TREG), mast cells, and tumor-associated macrophages (TAMs) determined by image cytometry analysis. Corresponding mIHC images were shown in bottom panels. Antibodies used for immunodetection are color-coded as shown. Boxes show the area magnified in the bottom panels. Magnification is shown. B, Ratios of cell percentages comparing intratumoral and adjacent nonmalignant regions are shown (N = 39). Bars, boxes, and whiskers represent median, interquartile range, and range, respectively. Statistical differences were determined via Wilcoxon signed-rank tests with false discovery rate (FDR) adjustments, with *P < 0.05, and ***P < 0.001 [Color figure can be viewed at wileyonlinelibrary.com]
performed on single sections of FFPE tissue, but is limited to immunodetection of adjacent tissue sections where immune cell phenotypes are identified by 1-3 antibody reactivity, typically; thus a comprehensive assessment of multiple cell types in single FFPE sections has not been published. mIHC approach circumvents these issues, and
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FIGURE 4 Immune complexity in papillary thyroid cancer (PTC) correlates with histopathological aggressiveness. A, Heat map indicating immune cell densities (cell counts per mm²) according to color scale (upper left) with a dendrogram of unsupervised hierarchical clustering, depicting myeloid/hypo-inflamed, and lymphoid-inflamed subgroups (M/H and L in bottom, respectively). B, Micrographs show multiplex immunohistochemistry (mIHC) findings in myeloid/hypo-inflamed, and lymphoid-inflamed subgroups in (A), showing high infiltration of myeloid and lymphoid cell populations, respectively. Antibodies and color annotations are shown at bottom. Scale bars = 500 μm (left) and 50 μm (right). C, Immune cell densities of lymphoid and myeloid cell lineages comparing hypo/myeloid-inflamed (n = 22) and lymphoid-inflamed (n = 17) subgroups. Bars, boxes, and whiskers represent median, interquartile range, and range, respectively. (D) Immune cell percentages quantified as a percentage of total CD45⁺ cells. Bars show median with interquartile range. E. The number of cases with pathological aggressiveness was evaluated, comparing the hypo/myeloid-inflamed and lymphoid-inflamed subgroups. Statistical differences determined by Chi-square test. F. Ratios of cell percentages comparing subgroups are shown. Data points in (D) and (F) indicate mean values per patient sample evaluated for the cell types shown. Bars show median with interquartile range. Statistical differences in (C), (D), and (F) were determined via Kruskal-Wallis tests with FDR adjustments, with *P < 0.05, **P < 0.01, and ***P < 0.001 [Color figure can be viewed at wileyonlinelibrary.com]
Thus, clinical interpretations of our data still require future studies involving larger cohorts and comprehensive ROI selections to assess TiME composition and disease aggressiveness in clinical perspective.

Despite these limitations, the present study demonstrates an initial glimpse into comprehensive leukocyte profiling present in the TiME of PTC and yield three previously unreported findings: first, the TiME of PTC is myeloid inflamed relative to adjacent nonmalignant tissue; second, tumors with a more myeloid hypo-inflamed TiME phenotype were more likely to demonstrate aggressive pathological features as compared to PTCs with a more lymphoid-dominant TiME; and third, while BRAF V600E mutant PTCs were not consistently more pathologically aggressive, they did demonstrate increased mast cell infiltration and decreased CD8+ T cell:TREG ratios, indicating that BRAF mutations may contribute to the relatively “immunosuppressed” TiME phenotype. Overall, these findings support the hypothesis that tumor-immune interactions in PTC reflect an important factor in disease pathogenesis and outcomes, and are consistent with recent reports in colon carcinoma,26,27 head and neck squamous cell carcinoma, non-small cell lung carcinoma, urothelial carcinoma, and other malignancies,28–31 where presence, location, and functional status of CD8+ T cells correlates with disease outcome. Although myeloid cell phenotype and presence was not considered in these other studies, as myeloid-targeted immune therapeutics enter the clinical arena,3,14 it is clear that deep characterization and understanding of both arms of the immune system must be considered for efficient and improved patient stratification, as well as for relieving T cell-suppressive mechanisms ascribed to various myeloid cell and B cell subsets common to TiMEs in solid tumors, including PTC.

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FIGURE 5  BRAF V600E mutation is associated with high mast cell infiltration. A, The number of cases with pathological aggressiveness was evaluated, comparing BRAF V600E mutation vs wild-type status revealing that BRAF mutation status did not correlate with pathological aggressiveness. Statistical difference was determined by Chi-square test. B, Mast cell densities comparing BRAF wild-type (WT, n = 22) vs mutation (MT, n = 17) status. Statistical significance was determined by Kruskal-Wallis tests, with **P < 0.01. C, Immune cell percentages were quantified as a percentage of total CD45+ cells, comparing BRAF wild-type (WT, n = 22) vs mutation (MT, n = 17) status. Data points in (C) indicate mean values per patient sample evaluated for mast cell density. Bars, boxes, and whiskers represent median, interquartile range, and range, respectively. Statistical significance was determined by Kruskal-Wallis tests with FDR adjustments.
Up To Cancer - Lustgarten Foundation Pancreatic Cancer Convergence Dream Team Translational Research Gran. A patent application related to the methodology described in the present work has been filed by T.T. and L.M.C.

CONFLICT OF INTEREST

No conflict of interest.

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REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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