Immune landscape of papillary thyroid cancer and immunotherapeutic implications

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Abstract

Although papillary thyroid cancer (PTC) is curable with excellent survival rate, patients with dedifferentiated PTC suffer the recurrence or death. As cancer immune escape plays a critical role in cancer progression, we aimed to investigate the relationship between differentiation and immune landscape of PTC and its implications for immunotherapy. Using The Cancer Genome Atlas data, we estimated the immune cell enrichment scores and overall immune infiltration, ImmuneScore, to characterize the immune landscape of PTC. Thyroid differentiation score (TDS) was calculated from 16 thyroid function genes. We demonstrated that ImmuneScore had a significant negative correlation with TDS, and BRAFV600E+ tumors showed significantly low TDS and high ImmuneScore. Enrichment scores of myeloid cells and B-cells were negatively correlated with TDS, while those of plasma cells were positively correlated with TDS. In addition, the association between TDS, ImmuneScore and immunosuppressive markers (CTLA-4, PD-L1, HLA-G) were evaluated according to BRAFV600E status. All immunosuppressive markers expression had a significant negative correlation with TDS, and they were significantly higher in BRAFV600E+ status. Subgroups were divided by median values of TDS and ImmuneScore, and immunosuppressive markers of these subgroups were compared. The immunosuppressive markers expression was the highest in high ImmuneScore and low TDS subgroup. Furthermore, ImmuneScore had a significant association with recurrence-free survival, irrespective of clinicopathologic factors including BRAFV600E status. These findings based on gene expression data illuminate the immune landscape of PTC and its association with TDS, immunosuppressive markers and recurrence. Our results would be extended to investigate immunotherapeutic approaches in PTC.

Key Words
- papillary thyroid cancer
- tumor microenvironment
- immune landscape
- cancer immunoediting
- next generation sequencing

Introduction

Thyroid cancer is the most common endocrine malignancy, and its incidence has increased almost 3-fold over the past decades (Chen et al. 2009, Siegel et al. 2016). Papillary thyroid cancer (PTC) is the predominant histologic subtype (~90%) and is usually curable with a 10-year survival rate of greater than 90% (Haugen et al. 2016). However, some of PTC dedifferentiate into more aggressive thyroid cancers, so that 10% of patients...
experience the recurrence of PTC, and 5–10% of patients experience distant metastasis (Sipos & Mazzaferri 2010). Most of the differentiated PTC responds well to current treatment options including surgery, and radioactive iodine therapies, alone or in combination; however, dedifferentiated tumors exhibit 5-year survival rate of only 50–55% (Sherma 2003).

The close relationship between the tumor microenvironment (TME) and cancer cells are well demonstrated. Especially, the dynamic interaction between immune cells within TME and cancer cells contributes to all stages of carcinogenesis from the early state of tumorigenesis to cancer progression and metastasis (Crespo et al. 2013, Joyce & Fearon 2015). It is now well recognized that immune cells within TME could recognize and eliminate cancer cells; however, they also modulate host immune system to escape their surveillance by change of immune cell composition including recruitment of suppressor immune cell populations, downregulation of neantigen recognition, and expression of immune-inhibitory ligands, known as cancer immunoediting (Schreiber et al. 2011). To this end, modulations of the immune system within TME are of enormous interest among oncologists and cancer immunologists, and it leads to the development of the immunotherapy. In recent years, some immunotherapeutic agents including immune checkpoint inhibitors have demonstrated promising clinical results in patients with metastatic melanoma, non-small-cell lung carcinoma, and renal cell carcinoma (Postow et al. 2015). In the light of these successes, there has been interest in the investigation of cancer immunotherapeutics across other solid cancers, especially for a broad range of therapeutically refractory cancers including dedifferentiated PTC (French et al. 2017).

Here, we aimed to investigate the immune landscape of PTC and its association with differentiation and to evaluate the potential immunotherapeutic implications. To gain insights into tumor immune landscape according to differentiation of PTC, we performed integrative immune cell types enrichment analysis using gene expression profiles. We also assessed the association between immune landscape and the most common genetic alteration of PTC, the BRAFV600E mutation. From the comprehensive analysis, we focused on the clinical utility of immune landscape on prognostic stratification and potential implication as a biomarker reflecting immune microenvironment.

**Materials and methods**

**The preprocessing step for transcriptome and clinical data**

We used mRNA transcriptome data of PTCs from The Cancer Genome Atlas projects (TCGA) (Cancer Genome Atlas Research Network 2014). Using ‘TCGABiolinks’ R package (Colaprico et al. 2016), we downloaded the level three RNA sequence data of PTC from TCGA data portal (https://portal.gdc.cancer.gov/) on Oct 12th, 2017, obtained with Illumina HiSeq RNASeqV2 (Illumina, San Diego, CA, USA). We then normalized mRNA transcripts using ‘TCGAAnalyze_Normalization’ function and the expression data of 19303 genes from 505 samples were used to estimate two key variables – immune cell enrichment score and thyroid differentiation score – for further analysis.

The corresponding clinicopathological data from TCGA dataset including stage, follow-up time and time of recurrence were downloaded from the website of The cBioPortal for Cancer Genomics (http://www.cbiportal.org/) on Oct 12th, 2017 (Gao et al. 2013). PTC recurrence was defined based on the histological/cytological/radiographical/biochemical criteria of new thyroid cancer tumor-related events. More specifically, patients with any types of new tumor events, including locoregional recurrent tumor, distant recurrent tumor and biochemical recurrent tumor, were identified as having disease recurrence (Haugen et al. 2016). The recurrence time was defined as the time interval from the date of disease recurrence or progression to the date of initial pathologic diagnosis. Patients who had no new thyroid tumor were treated as having no disease recurrence. The duration to the last follow-up was defined as time interval from the date the patient was seen or contacted at follow-up to the date of initial pathologic diagnosis.

**Cell types enrichment analysis**

The xCell tool (http://xcell.ucsf.edu/), a gene-signature-based method for inferring cell types from tissue transcriptome profiles, was used to evaluate the heterogeneous cellular landscape of TME of PTC (Aran et al. 2017). Using the normalized mRNA transcriptome data of 505 PTC samples, the xCell tool inferred immune and stroma cell types enrichment scores of TME. The log2-transformed composite score of overall immune cells, ImmuneScore, was also obtained. More
specifically, to obtain ImmuneScore, the sum of cell enrichment scores of B-cells, CD4+ T-cells, CD8+ T-cells, dendritic cells, eosinophils, macrophages, monocytes, mast cells, neutrophils and NK cells was calculated and log2-transformed.

**TDS calculation**

Loss of expression of thyroid differentiation markers is one of the hallmarks of advanced PTC and has profound consequences for the clinical management of these patients. TDS, which was developed to quantify relationships between thyroid differentiation and other genetic and clinicopathologic features by the TCGA group (Cancer Genome Atlas Research Network 2014), was calculated across all 505 PTC samples. The mRNA expression level of sixteen thyroid function genes (DIO1, DIO2, DUOX1, DUOX2, FOXE1, GLIS3, NKX2-1, PAX8, SLC26A4, SLC5A5, SLC5A8, TG, THR, THR, TPO, TSHR) were used to derive the TDS. The log2-normalized RSEM values, transcript values quantified by RSEM software package (Li & Dewey 2011), were first centered at the median across samples, and then summed across the 16 genes for each sample.

**Statistical analysis**

The correlation between ImmuneScore and TDS was calculated using the Pearson’s correlation. Correlation coefficients between ImmuneScore and TDS were calculated for BRAFV600E+ and BRAFV600E− groups, and they were statistically compared using Fisher r-to-z transformation. We compared ImmuneScore and TDS according to the BRAFV600E mutation by the Student’s t-test.

The correlation between immune cell enrichment scores and TDS was conducted by the Spearman’s correlation test as the distribution of each immune cell enrichment score was skewed. The Spearman’s correlation coefficients of BRAFV600E-positive and -negative groups were compared by a permutation test. Subjects were randomly permuted into two groups and the distribution of the difference of correlation coefficients was obtained. Statistical significance of the difference of correlation coefficients between two groups was directly calculated by the comparison with the distribution obtained by iteratively permuted samples. The multiple testing corrections were performed using the Bonferroni’s method. Each immune cell enrichment score of subgroups according to the BRAFV600E mutation was statistically compared using Mann–Whitney test with Bonferroni’s multiple testing corrections.

Expression of immunosuppressive molecular markers, CTLA-4, PD-L1 and HLA-G, was statistically compared according to TDS and the BRAFV600E mutation status. The correlation between TDS and immunosuppressive markers was conducted by the Pearson’s correlation test. We further divided subjects based on the median value of TDS and ImmuneScore to characterize the expression pattern of immunosuppressive markers. The expression of immunosuppressive markers between four groups was compared by one-way ANOVA, followed by the post hoc Tukey’s test. The expression level of immunosuppressive markers was also compared according to the BRAFV600E mutation status. The log2-transformed expression value of immunosuppressive markers was used for the aforementioned analysis.

The recurrence-free survival (RFS) rate was depicted with the Kaplan–Meier’s method and compared with the log-rank test. The survival analyses were performed in all subjects and in subgroups defined by T-stage, the existence of lymph node metastasis and the presence of the BRAFV600E mutation. Prognostic property of ImmuneScore for RFS was evaluated by univariate and multivariate Cox regression analysis. For all survival analysis, the subjects were divided into two groups based on the median value of ImmuneScore and RFS was compared between two groups.

**Results**

**Baseline characteristics**

The subjects were 369 female (73.1%) and 136 male (26.9%) with a mean age of 47.8 ± 15.8 years (range, 15.4–89.6). The distribution of stage according to the criteria established by the American Joint Committee on Cancer was as follows: T-stage distribution – T1 (28.9%), T2 (33.9%), T3 (33.3%), and T4 (3.9%); N stage distribution – N0 (50.8%), and N1 (49.2%); M stage distribution – M0 (96.9%) and M1 (3.1%). The BRAFV600E mutation was identified in 235 patients (46.5%). Median recurrence-free time was 14.3 months (0.0–158.7), and 28 subjects (6.6%) among 423 subjects with available survival data experienced recurrence. The detailed baseline characteristics are listed in Table 1.

**ImmuneScore associated with TDS and the BRAFV600E mutation**

ImmuneScore had significant negative correlation with TDS (r = −0.44, P < 1 × 10−15; Fig. 1A). The negative correlation was conserved independent of the presence
of the BRAF<sup>V600E</sup> mutation, but the correlation was significantly stronger for BRAF<sup>V600E</sup>− group (r = −0.18 and P = 0.005 for BRAF<sup>V600E</sup>− group; r = −0.39 and P < 1 × 10<sup>−10</sup> for BRAF<sup>V600E</sup>− group; P = 0.01 for comparison of correlation coefficients, Fig. 1A). ImmuneScore was significantly higher in BRAF<sup>V600E</sup>− group (BRAF<sup>V600E</sup>− group −4.41 ± 1.24 vs BRAF<sup>V600E</sup>− group −5.57 ± 1.84; P < .001; 95% CI 0.88–1.44; Fig. 1B). Moreover, TDS was significantly lower in BRAF<sup>V600E</sup>− group (BRAF<sup>V600E</sup>− group −0.70 ± 0.78 vs BRAF<sup>V600E</sup>− group 0.50 ± 1.15; P < 1 × 10<sup>−15</sup>; 95% CI 1.03–1.38; Fig. 1C). Conclusively, high ImmuneScore, which represents enriched immune cells in TME, were associated with low TDS and the BRAF<sup>V600E</sup> mutation.

**Immune cell enrichment scores according to TDS**

In order to investigate immune landscape according to PTC profiles, individual immune cell enrichment scores according to TDS was visualized by a heatmap (Fig. 2A). We further analyzed the correlation between individual immune cell enrichment scores and TDS (Fig. 2B). There was a strong negative correlation between TDS and myeloid cells such as macrophage, dendritic cells and mast cells (r = −0.35, −0.49 and −0.35, respectively; Bonferroni corrected P < 0.05). This relationship was only demonstrated in BRAF<sup>V600E</sup>− group (r = −0.37, −0.49, and −0.233, respectively). Plasma cell showed strong positive correlation with TDS, especially in BRAF<sup>V600E</sup>− group (r = 0.34 for all samples and r = 0.44 for BRAF<sup>V600E</sup>− group; Bonferroni corrected P < 0.05), while B-cells showed significant negative correlation with TDS (r = −0.34 for all samples). Statistical comparison showed that correlation coefficients of plasma cells (P < 0.001), macrophages (P = 0.004), dendritic cells (P = 0.001) and mast cells (P < 0.01) were significantly different between BRAF<sup>V600E</sup>− and BRAF<sup>V600E</sup>− groups.

In addition, individual immune cell enrichment scores were compared according to the presence of the BRAF<sup>V600E</sup> mutation. Myeloid cells (macrophage, dendritic cell, neutrophil and mast cell), B-cell and regulatory T-cells were significantly higher in BRAF<sup>V600E</sup>− group and plasma cells were significantly higher in BRAF<sup>V600E</sup>− group (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Briefly, relatively low differentiation of PTC was associated with higher immune infiltration particularly for myeloid subtypes and relatively lower effector lymphocytes such as plasma cells.

**TDS and expression of immunosuppressive markers**

We investigated whether TDS is associated with expression of immunosuppressive markers including CTLA-4, PD-L1 and HLA-G. TDS was significantly associated with decreased expression of immunosuppressive markers (Fig. 3A, B and C). TDS demonstrated strong negative correlation with CTLA-4 (r = −0.47, P < 1 × 10<sup>−15</sup>), PD-L1 (r = −0.33, P < 1 × 10<sup>−13</sup>) and HLA-G (r = −0.50, P < 1 × 10<sup>−15</sup>). The immunosuppressive markers were significantly different according to the BRAF<sup>V600E</sup> mutation status (Fig. 3D, E and F). The three markers were significantly higher in BRAF<sup>V600E</sup>− group (t = −6.45, P < 1 × 10<sup>−9</sup> for CTLA-4; t = −7.66, P < 1 × 10<sup>−13</sup> for PD-L1; t = −5.54, P < 1 × 10<sup>−7</sup> for HLA-G).

Immunosuppressive markers were compared between four groups divided by the median values of TDS and ImmuneScore (Fig. 4A). There was a significant effect
of the group on immunosuppressive markers ($F = 134.9, P < 1 \times 10^{-15}$ for CTLA-4, $F = 401.9, P < 1 \times 10^{-15}$ for PD-L1 and $F = 90.5, P < 1 \times 10^{-15}$ for HLA-G; Fig. 4B, C and D). Post hoc analyses revealed that all between-group differences were statistically significant except CTLA-4 expression between groups 1 and 3 (Fig. 4B, C and D). Specifically, expression of PD-L1 and HLA-G was significantly higher in the group 3 (high ImmuneScore and low TDS) than the other groups (post hoc Tukey multiple comparison $P < .001$; Fig. 4C and D).

**Recurrence-free survival analysis**

In all patients, the high ImmuneScore groups demonstrated worse RFS than the low ImmuneScore group ($P = 0.007$; Fig. 5). We further analyzed whether ImmuneScore was associated with RFS in subgroups according to other clinical variables (Supplementary Fig. 2). ImmuneScore was significantly associated with RFS in the subgroup with T1 and T2 stages and the subgroup with lymph node metastasis ($P = 0.04$ and $0.007$, respectively). In addition,
there was a trend of the association between ImmuneScore and RFS regardless of the BRAFV600E mutation status. (Supplementary Fig. 3; \(P = 0.04\) for BRAFV600E− group and \(P = 0.16\) for BRAFV600E+ group). Table 2 shows the hazard ratios of the impacts of ImmuneScore on PTC recurrence. The high ImmuneScore group alone demonstrated significant impact on PTC recurrence (HR = 2.66; CI 1.17–60.5; \(P = 0.019\)) and maintained the significance after adjustment for age and gender (HR = 2.82; CI 1.23–6.47; \(P = 0.012\)). After the additional adjustment for T-stage and lymph node metastasis, the high ImmuneScore group showed a trend of worse RFS with borderline significance (HR = 2.31; CI 0.94–5.70; \(P = 0.069\)).

**Discussion**

In this study, we estimated immune cell enrichment scores and differentiation of PTC using gene expression data and analyzed the immune landscape of PTC in accordance with TDS and BRAFV600E mutations. As a result, the BRAFV600E mutation was associated with increased overall immune infiltration signature, ImmuneScore. In addition, ImmuneScore was positively correlated with TDS. The principle of immunosurveillance of tumors can be summarized by tumor regression mediated by immunological effector cells, while progression of cancers mediated by immunosuppressive cells (Schreiber et al. 2011, Landa et al. 2016). In terms of the immunoediting in accordance with oncogenesis, thyroid cancers with relatively worse prognosis can constitute TME that enables escape from immunosurveillance by evolving into genetically modified cancer cells. In this regard, we showed that immune cell enrichment scores were increased in the PTCs with low TDS and BRAFV600E mutation. This result corresponds to the recent report of the association between the BRAFV600E mutation in PTC and suppressive immune cell infiltration (Angell et al. 2014). To further elaborate, we elucidated specific types of immune cell populations contributed to immunosuppressive TME. Enrichment scores of myeloid cells including dendritic cells, macrophages and mast cells were increased in PTC with low TDS and PTC with the BRAFV600E mutation. These findings were consistent with some previous experimental studies aiming at the association between each cell type and aggressiveness of PTC (Hilly et al. 2013, Yu et al. 2013, Angell et al. 2014, Tran Janco et al. 2015). Although dendritic cells are rarely found in normal thyroid tissues, they are increased in PTC and often adopt immature phenotypes, which inhibit immune responses (Hilly et al. 2013, Tran Janco et al. 2015). Tumor-associated macrophages are also associated with tumor invasiveness and decreased survival (Ryder et al. 2008). Of note, TDS was positively correlated with plasma cells, while...
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it was negatively correlated with overall B-cell population. It implies that immature immune cells may be increased and effector cells such as plasma cells may be reduced as an immune escape mechanism in PTC with low TDS.

As some PTCs develop into invasive disease and eventually radiiodine-refractory cancer according to dedifferentiation, novel therapies for these PTCs are necessary. Recently, antitumor efficacy of immune checkpoint therapy has been observed in a wide variety of tumors (Sharma & Allison 2015). Thus, there has been strong interest in advanced PTCs as one of the most promising candidates for immune checkpoint therapy (French et al. 2017). To optimize this therapeutic approach, companion diagnostics should be developed to identify which patients are likely to achieve benefit (Topalian et al. 2016). In this regard, multiple biomarkers have been suggested for predicting the response, which includes PD-L1 expression, tumor-infiltrating lymphocytes, TME population and immune-related gene signatures (Topalian et al. 2016). Though TCGA data, we used in this analysis, excluded clinically aggressive thyroid cancers, we aimed to investigate whether there is a difference in immunosuppressive markers expression, including potential immunotherapeutic targets such as PD-L1 and HLA-G, according to TDS and BRAFV600E status. Our results demonstrated increased immunosuppressive markers expression according to low TDS and BRAFV600E+. This was consistent with previous immunohistochemistry results, which showed BRAFV600E status closely associated with regulatory T-cells and immunosuppressive macrophage components as well as immunosuppressive markers including PD-L1 (Angell et al. 2014). Therefore, these results could provide insights that tumors with low TDS and BRAFV600E+ might have potential to have therapeutic efficacy to immune checkpoint blockades.

ImmuneScore of PTC was a prognostic marker in all patients as well as subpopulation according to

Figure 4
Distribution of immunosuppressive markers according to TDS and ImmuneScore. (A) All samples were divided into four groups based on the median value of TDS and ImmuneScore. Four groups are indicated in different shapes. (B, C and D) There was a significant effect of the group on the expression of immunosuppressive markers (F = 134.9, P < 1 × 10^-15 for CTLA-4, F = 401.9, P < 1 × 10^-15 for PD-L1 and F = 90.5, P < 1 × 10^-15 for HLA-G). The expression of PD-L1 and HLA-G was significantly higher in the group 3 (high ImmuneScore and low TDS) than other groups in the post hoc Tukey analysis (B and C). (NS, not significant; **P < 0.01; ***P < 0.001.)
the BRAF<sup>V600E</sup> mutation status and tumor stages. In multivariate Cox regression analysis, ImmuneScore was associated with recurrence risk after the adjustment of clinical prognostic factors such as age, sex, T-stage and lymph nodes metastasis. Hence, we think ImmuneScore-based prognostic stratification can hold promise for improving management of PTC by identifying patients with high recurrence risk. Furthermore, ImmuneScore might be utilized for determining whether intensive adjuvant therapies and recurrence monitoring are needed in addition to the BRAF<sup>V600E</sup> mutation exam, as it can stratify patients regardless of the BRAF<sup>V600E</sup> status. Currently, the mutational testing for BRAF has been actively reflected in the guideline of thyroid nodule management as well as risk stratification of differentiated thyroid cancer (Melck et al. 2010, Haugen et al. 2016). ImmuneScore might be used as a prognostic biomarker that reflects novel molecular targets related to immune checkpoint blockades. A recent report showed that PD-L1 expression, an immunosuppressive marker, was associated with recurrence of PTC (Shi et al. 2017). It corresponds to our results in terms of immunosuppressive TME related to higher recurrence risk. Considering multiple predictive biomarkers have been suggested for immune checkpoint blockades (Topalian et al. 2016), ImmuneScore as well as various molecular markers including PD-L1 should be comprehensively reviewed in terms of prognostic and predictive biomarkers as a future work.

In spite of investigating the close association between immune TME and TDS, and their immunotherapeutic implications in PTC in our study, the comprehensive multimodal analysis including transcriptome, and histopathologic data should be performed to support our results (Galon et al. 2014). The key variables used in the analysis are immune cell enrichment score and TDS, and both were estimated based on mRNA transcriptome data. Although the immune cell enrichment scores and TDS are good surrogate indicators for immune cell population in TME and differentiation of PTC respectively in the previous studies (Cancer Genome Atlas Research Network 2014, Aran et al. 2017), future research with histopathologic examination of TME and tumor differentiation will elucidate our results and promote clinical utility as biomarkers. Furthermore, mRNA transcriptome-based analyses need to be further validated in PTC samples as a future work. Investigation of tumor differentiation and ImmuneScore in prospectively enrolled refractory PTC patients may provide insights into immunotherapeutic implications and prognostic stratification based on immune landscape and tumor differentiation.

In conclusion, the immune landscape of PTC is prone to constitute immunosuppressive TME by increasing myeloid cells and decreasing effector cells according to low TDS and the BRAF<sup>V600E</sup> mutation. Because high immunosuppressive markers were associated with PTC with low TDS and the BRAF<sup>V600E</sup> mutation, they might be a potential candidate for the investigation of immunotherapeutic approaches. Furthermore, high ImmuneScore was associated with high immunosuppressive markers expression as well as worse RFS, so that it might have potential as a prognostic and predictive biomarker of PTC. We propose that immune landscape characterization of PTC can be an integral correlative aspect of the ongoing immunotherapeutic investigation and plays an important role in PTC management.

### Table 2 Hazard ratio of ImmuneScore for the recurrence of PTC.

<table>
<thead>
<tr>
<th>Hazard ratio of high ImmuneScore group (95% CI)</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Unadjusted</td>
<td>0.019</td>
</tr>
<tr>
<td>Adjusted for age and gender</td>
<td>0.015</td>
</tr>
<tr>
<td>Adjusted for age, gender, T-stage, and lymph node metastasis</td>
<td>0.069</td>
</tr>
</tbody>
</table>

Cl, confidence interval.
Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-17-0532.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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