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Transcriptomic signatures associated with autoimmune thyroiditis in papillary thyroid carcinoma and cancer immunotherapy-induced thyroid dysfunction

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ABSTRACT

Up to 20% of patients treated with anti-PD-1/PD-L1 inhibitors suffered from thyroid dysfunctions, yet the mediators associated with their occurrence remain unclear. The increasing coincidence of papillary thyroid carcinoma (PTC) with Hashimoto thyroiditis (HT) and the high vulnerability of thyroid to immunotherapy motivated us to discover the similarities and their underlying transcriptomic basis. Clinical characteristics analysis of 468 PTC patients from two independent cohorts and meta-analysis of 22,155 PTC patients unveiled a strong negative association between HT and recurrence in PTC patients. Transcriptome analysis of both cohorts showed PTC patients with HT were enriched in macrophages, $CD8^+$ and $CD4^+$ cytotoxic T cells, which was further validated by single-cell transcriptome analysis of 17,438 cells from PTC patients, and $CD8^+$ T cells were correlated with disease-free survival of PTC patients. In both cohorts and single-cell dataset, elevated expression of PD-1-related genes was observed in the HT group, and *CD3D* appeared to be a target for enhancing the activation of $CD8^+$ T cells. Correlation analysis of 3,318 thyroid adverse events from 39,123 patients across 24 tumor types and molecular signatures demonstrated similar signatures associated with autoimmune thyroiditis in PTC and thyroid immune-related adverse events (irAEs), and several multi-omics signatures, including signatures of CD8A and $CD8^+$ T cells, showed positive associations with the odds ratio of thyroid irAEs. Our results unveil shared molecular signatures underlying thyroid dysfunction between patients receiving immunotherapies and PTC patients suffering from HT, which may shed light on managing the adverse events during cancer immunotherapy.

1. Introduction

Immune checkpoint blockade targeting programmed cell death-1 (PD-1)/PD-L1 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) has revolutionized cancer treatment, and it has been used in the treatment of various cancer malignancies [1,2]. However, a spectrum of immune-related adverse events (irAEs) have been characterized during immune checkpoint inhibitor (ICI) therapy, which restricts its clinical application [3–5]. Thyroid dysfunction has now emerged as one of the most common adverse events, with an incidence as high as 15–20% across studies during anti-PD-1 therapy [6–8]. Thyroid dysfunction typically manifests as destructive autoimmune thyroiditis, ranging from transient mild or moderate thyrotoxicosis to restoration of euthyroidism or progression to hypothyroidism within three months after initiation of immunotherapy [9]. Limited by the lack of an appropriate model system, the etiology and molecular processes associated with the occurrence of this type of adverse event remain unclear.

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Hashimoto thyroiditis (HT), also known as chronic lymphocytic thyroiditis, is one of the most common autoimmune thyroid disorders [10]. The prevalence of this disorder has increased in recent years, affecting 0.1–5% of all adults in Western countries [11]. It can give rise to the loss of immunological tolerance, with the subsequent immune system attacking the thyroid tissue and the appearance of autoimmune disease [12]. Although cancer and autoimmunity have been deemed to be two opposite extremes of immune responses, several studies have revealed that papillary thyroid cancer (PTC), another frequently occurring thyroid pathology with an incidence tripling over the past three decades [13–16], coexists with autoimmune thyroid diseases such as HT [17]. The mean coexistence rate between HT and PTC in epidemiologic studies was approximately 23% [18]. However, there remain some different views regarding the relationship of the coexistence [19]. A number of studies have reported that HT may promote the occurrence of PTC [12,20–22]. Patients with PTC coexisting with HT were predominantly multifocal compared to PTC patients without HT [20], and the metastasis of lymph nodes in PTC concomitant with HT was more frequent than that in PTC patients without HT [23]. On the other hand, some reports demonstrated that PTC with HT was less aggressive and had a lower rate of recurrence, small tumor size, and lower frequency of distant metastases, and the survival rate was better than that of those without HT [10,12,20–22,24–26]. It is worth noting that the exact molecular basis underlying the clinical outcome of the co-occurrence of PTC and HT remains poorly characterized. The growing coincidence of PTC with autoimmune thyroid disorders provides an optimal model to investigate the interaction between cancer and autoimmunity at this gland, and the high vulnerability of the thyroid to immune checkpoint blockade therapy motivated us to discover the biological basis underlying thyroid IRAEs during cancer immunotherapy based on this model.

To our knowledge, there have not been any reports about the similarities/differences or their underlying molecular basis between PD-1-induced thyroiditis and thyroiditis combined with thyroid carcinoma. Therefore, the aim of this work was to explore 1) whether there was a difference in recurrence between PTC with (HT) and without HT (Non-HT); 2) why patients with PTC combined with HT had a better prognosis than those without HT; and 3) the molecular links between immunotherapy-induced thyroid dysfunction and thyroiditis combined with thyroid carcinoma.

2. Materials and methods

2.1. Collection and analysis of clinical data

A total of 621 primary PTC patients who underwent thyroid surgery from two independent cohorts were collected from published studies and the TCGA database [27,28]. The diagnosis of each tumor was determined by specialized pathologists based on the pathological image of thyroidectomy specimens, and the TNM Classification of Malignant Tumors was used for PTC staging. One hundred and twenty-five PTC patients from the Korean cohort and 343 PTC patients from the TCGA-THCA cohort with clear annotation of "history of thyroiditis" were included in this study (**Supplementary Table S1**), and the prognosis of recurrence was defined according to the status of "progression-free interval event" [29]. In addition, the original articles were searched in PubMed using the following terms: (Hashimoto thyroiditis OR Hashimoto disease OR chronic lymphocytic thyroiditis OR Hashimoto struma OR Hashimoto syndrome) AND (thyroid carcinoma OR thyroid cancer OR thyroid tumor OR thyroid neoplasm) AND (cohort OR prospective OR case-control). Articles were only included if they satisfied the criteria shown in **Supplementary Figure S1A**, and a total of 20 studies including 22,155 PTC patients, of whom 4,504 had HT, satisfied the inclusion criteria were gathered for this meta-analysis. Meta-analysis was then conducted by the R package "meta" [30]. The sensitivity analysis for publication bias in this meta-analysis was conducted using "metabias" function of R package "meta", and no significant publication bias was observed in this meta-analysis (P = 0.1454; **Supplementary Figure S2**).

2.2. Analysis of transcriptome sequencing data and identification of differentially expressed genes

We collected the transcriptome sequencing data of 125 Korean PTC patients from the ENA database with accession number PRJEB11591 [28]. After cleaning adapters and low-quality bases, sequencing reads were mapped to the human reference genome (hg19) by STAR (https://github.com/alexdobin/STAR) with default parameters. Next, the read count data of each protein-coding gene were obtained using HTSeq (https://github.com/htseq/htseq) according to the annotation of GENCODE (V22). Additionally, we downloaded a read matrix of 343 PTC patients from the TCGA-THCA cohort [27] and grouped the matrices into HT and Non-HT groups according to the clinical information. Then, the differentially expressed genes between the HT and Non-HT groups were obtained using the edgeR package with a false discovery rate (FDR) of 5% after taking into account the differences of 242 expression profiles between paired PTC tumor and their adjacent normal tissues [14]. The enrichment analysis of the differentially expressed genes obtained by both cohorts was conducted using the R package of Enrichr [31].

2.3. Abundance estimation of immune cells and survival analysis

We applied two algorithms, ESTIMATE [32] and xCELL [33], to calculate immune scores based on transcriptome expression data. CIBERSORT [34], EPIC [35], MCP-counter [36], quanTIseq [37], xCELL [33], ImmuneCellAI [38] and TIMMER [39] were used to estimate the abundance of specific immune cells. Kaplan–Meier survival analysis was employed with the R survival and survminer packages.

2.4. Analysis of single-cell expression data

Single-cell expression data of six female PTC patients, including three with concurrent HT and three without HT, were collected from the Gene Expression Omnibus (GEO) with accession number GSE163203 [40]. The gene expression matrices were analyzed using the R package Seurat. We selected the top 2000 most variable genes to perform principal component analysis (PCA) and then used UMAP dimensionality reduction to summarize the principal components and identify major cell types.

2.5. Association between thyroid dysfunction and ICI therapy

We retrieved the safety data of individual patients from the US Food and Drug Administration Adverse Event Reporting System (FAERS) spanning from July 1, 2014 to December 31, 2021. We defined adverse effects of thyroid dysfunction as hypothyroidism, hyperthyroidism, thyroiditis and other thyroid hormone abnormalities according to a previous report [41] and collected thyroid dysfunction records after usage of anti-PD-1 inhibitors (pembrolizumab, nivolumab, cemiplimab) and/or anti-PD-L1 (avelumab, atezolizumab, durvalumab) from FAERS. In this group treated with anti-PD-1/PD-L1 inhibitors, we excluded patients who were also treated with anti-CTLA-4 inhibitors (ipilimumab, tremelimumab). The odds ratio (OR) of thyroid irAEs was calculated by comparing the proportion of reported thyroid irAEs for

anti-PD-1/PD-L1 inhibitors with that for all other inhibitors in the FAERS. Molecular signature datasets were downloaded from the GDC PanImmune Data Portal [42]. We adopted the approach of Jing *et al.* to evaluate the correlation between a specific molecular signature and the odds ratio of thyroid dysfunction [43].

3. Results

3.1. Lower recurrence rates for PTC with HT

To examine whether there exists a difference in recurrence between PTC with HT (HT) and without HT (Non-HT), we first analyzed the clinical data of 125 and 343 PTC patients from two independent cohorts [27,28], of whom 41 (32.8%) and 69 (20%) had HT, respectively. PTC with concomitant HT was observed to be negatively associated with the recurrence of PTC without HT in the THCA (*OR* = 0.24; 95% *CI* = 0.03–1; *P* = 0.038) (**Supplementary Table S1**) and the combined cohort (*OR* = 0.28; 95% *CI* = 0.053–0.91; *P* = 0.024) (Fig. 1**A**). To determine this observation, we then conducted a meta-analysis of the available studies through a PubMed search using the keywords listed in the Methods, which retrieved 553 publications (**Supplementary Figure S1A**). Finally, a total of 20 studies including 22,155 PTC patients, of whom 4,504 had HT, satisfied the inclusion criteria for this meta-analysis [10,18,21,22,26,28,44–55] (Fig. 1**B**). The sample sizes of each study ranged from 37 to 9,210 cases, and no significant inter-study heterogeneity was observed (I^2 = 9%, *P* = 0.35) (Fig. 1**B**). The overall risk of recurrence in PTC patients with HT (HT) was observed to be significantly lower than that in PTC patients without HT (Non-HT) (*OR* = 0.56; 95% *CI* = 0.48–0.65) (Fig. 1**B**). Taken together, these data demonstrated a strong negative association between HT and recurrence of PTC, suggesting a protective role of autoimmune thyroiditis in the prognosis of PTC patients.

3.2. Increased abundance of immune cells in PTC patients with Hashimoto thyroiditis

The tumor microenvironment (TME) is not simply a group of cancer cells but rather composed of various types of immune cells, which are critical for the growth or elimination of cancer cells [56]. To determine the association between the tumor microenvironment and the prognosis of PTC patients with autoimmune thyroiditis, we first examined immune cell abundance in PTC patients between the HT and Non-HT groups based on transcriptome data from two independent cohorts, THCA and Korea. In the Korea cohort, the average immune scores calculated by the two algorithms were both significantly higher in the HT group than in the Non-HT group (*P* = 0.00023 by ESTIMATE and *P* = 0.019 by xCELL) (Fig. 2**A, B**). The results were validated in another independent cohort, THCA (*P* = 0.0018 by ESTIMATE and *P* = 0.00032 by XCELL) (Fig. 2**A, B**). To further explore the difference in the abundance level of specific immune cells between the HT and Non-HT groups, 8 different tools were applied to detect the abundance of specific immune cells (**Supplementary Figure S1B**). Interestingly, in the HT group of both cohorts, the abundance of activated immune cells, including B cells and $CD8^+$ T cells, was markedly increased compared with that in the Non-HT group (Fig. 2**C, D**), which was supported by the predictions of different algorithms. Furthermore, we performed survival analysis based on the abundance of these identified immune cells. Kaplan–Meier results showed that a high level of $CD8^+$ T cells was associated with better disease-free survival of PTC patients in the TCGA-THCA cohort (**Supplementary Figure S3**). Importantly, some of the activated immune cells have already been proven to be positively associated with better survival of cancer patients who underwent ICI therapy. For instance, emerging evidence revealed that more activated $CD8^+$ T cell infiltration in patients who experienced immune checkpoint blockade could promote progression-free and overall survival [1,2,7,57,58]. Thus, the elevated abundance of these activated immune cells appears to be beneficial for PTC patients with autoimmune thyroiditis.

3.3. Altered PD-1/PD-L1 pathway in PTC patients with Hashimoto thyroiditis

To dissect genome-wide expression alterations associated with inflammation of PTC, we compared expression profiles between the HT and Non-HT groups in both cohorts after taking into account the differences between tumor and adjacent normal tissues and identified 215 differentially expressed genes shared by both cohorts. We observed that the majority of these inflammation-related genes were upregulated in the HT group (Fig. 3**A**). Protein–protein interaction analysis revealed that these interactions were densely connected around the modules that have been implicated in the functions of tumor escape and the development of autoimmune disorders [59,60] (Fig. 3**B**). These identified differentially expressed genes were enriched in immune-related categories, such as "T cell activation", "leukocyte cell–cell adhesion", "T cell differentiation" and "T cell proliferation" (Fig. 3**C**),

A

	HT	Non-HT	*P* value	OR	95%CI
N	110	358	-	-	-
Age	46.37	46.21	0.60	-	-
Gender			0.27	1.36	[0.81; 2.38]
Female	86(78.18%)	259(72.35%)			
Male	24(21.82%)	99(27.65%)			
Multifocal			0.0032	1.96	[1.24; 3.11]
Yes	55(50%)	125(34.92%)			
No	52(47.27%)	232(64.8%)			
NA	3(2.73%)	1(0.28%)			
TNM stage			0.58	-	-
I	73(66.36%)	217(60.61%)			
II	7(6.36%)	26(7.26%)			
III	24(21.82%)	80(22.35%)			
IV	6(5.45%)	34(9.5%)			
NA	-	1(0.28%)			
Recurrence			0.024	0.28	[0.053; 0.91]
Yes	3(2.73%)	33(9.22%)			
No	107(97.27%)	325(90.78%)			

B

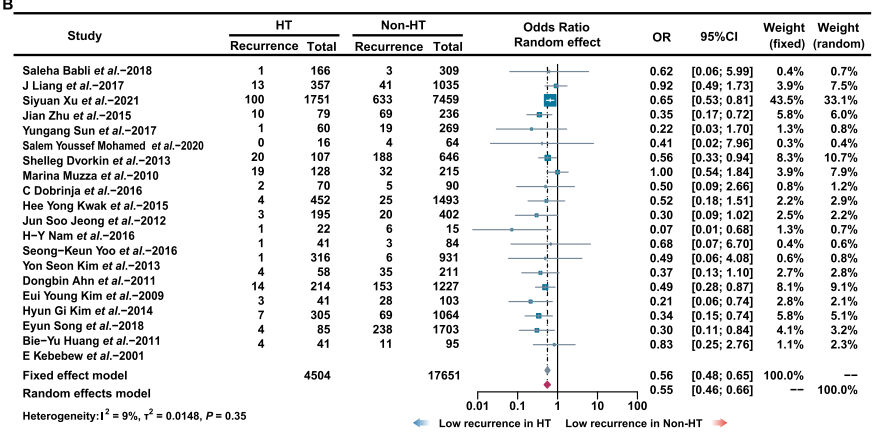


Fig. 1. Tumor recurrence difference between papillary thyroid cancer patients with (HT) and without (Non-HT) Hashimoto thyroiditis. (A) Clinical characteristics of the THCA-Korea combined cohort. (B) Forest plots summarizing the pooled risk ratio of the association between Hashimoto thyroiditis and recurrence of papillary thyroid cancer. OR, odds ratio; CI, confidence interval.

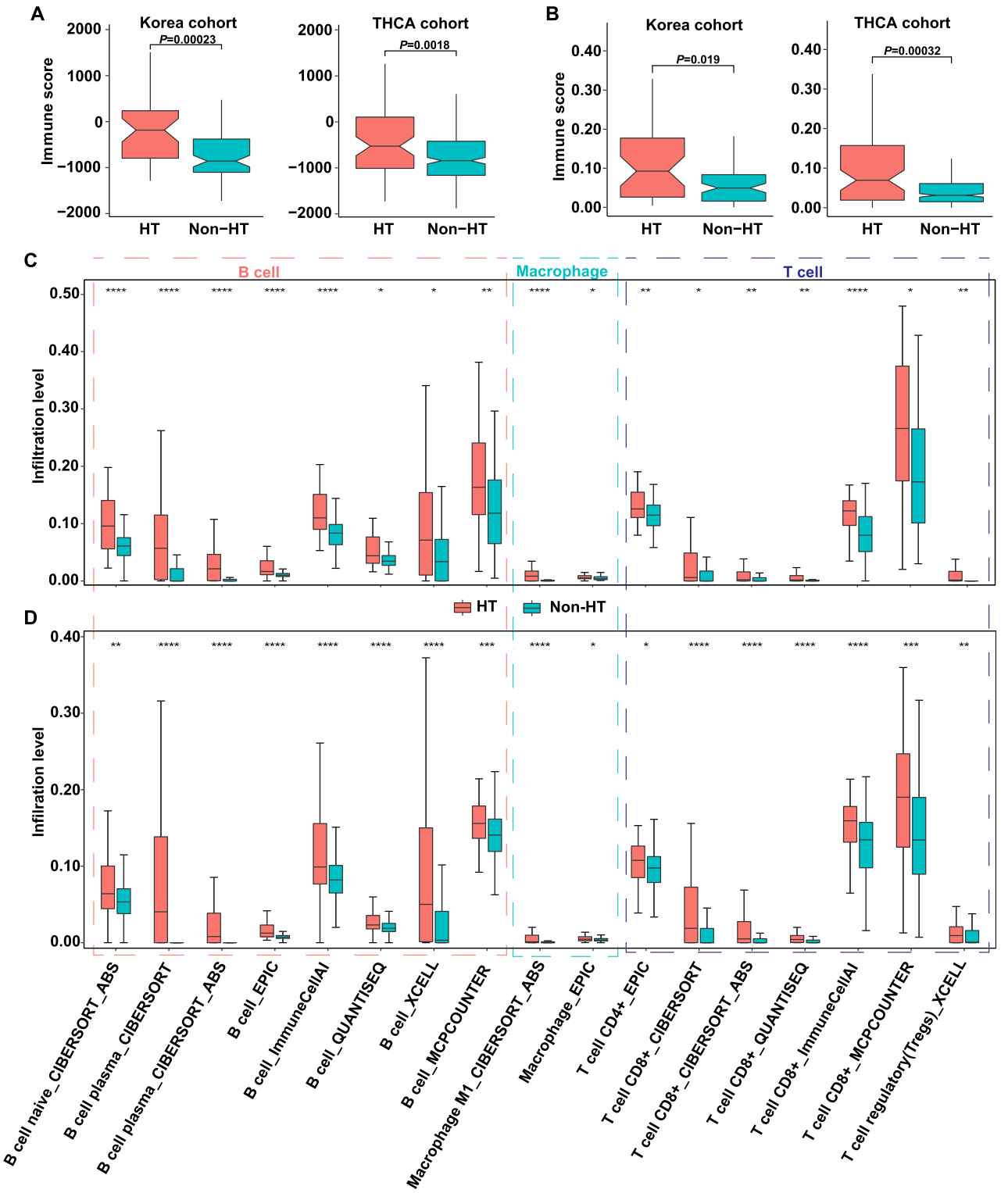


Fig. 2. Immune score and abundance of immune cells between PTC patients with HT and without HT (Non-HT) in the THCA and Korean cohorts. Immune scores of the HT and Non-HT groups in the THCA and Korean cohorts were measured by ESTIMATE (A) and xCELL (B), respectively. Comparison of the levels of immune cells between the HT and Non-HT groups in the Korea (C) and THCA (D) cohorts.

suggesting their involvement in the development of PTC patients with HT. Surprisingly, in addition to several immune-related pathways, we found that the "PD-L1 expression and PD-1 checkpoint pathway in cancer" category was enriched in our KEGG enrichment analysis (Fig. 3**D**). Several signaling pathways involved in the PD-1/PD-L1 axis [61], such as the "Toll-like receptor signaling pathway", "JAK-STAT signaling pathway" and "NF-kappa B signaling pathway", were also observed to be enriched. Subsequently, we utilized gene set enrichment analysis (GSEA) based on all transcriptome data instead of differentially expressed genes to estimate the biological activities associated with thyroiditis of PTC patients and showed elevated activity of PD-1-mediated T cell signaling in HT

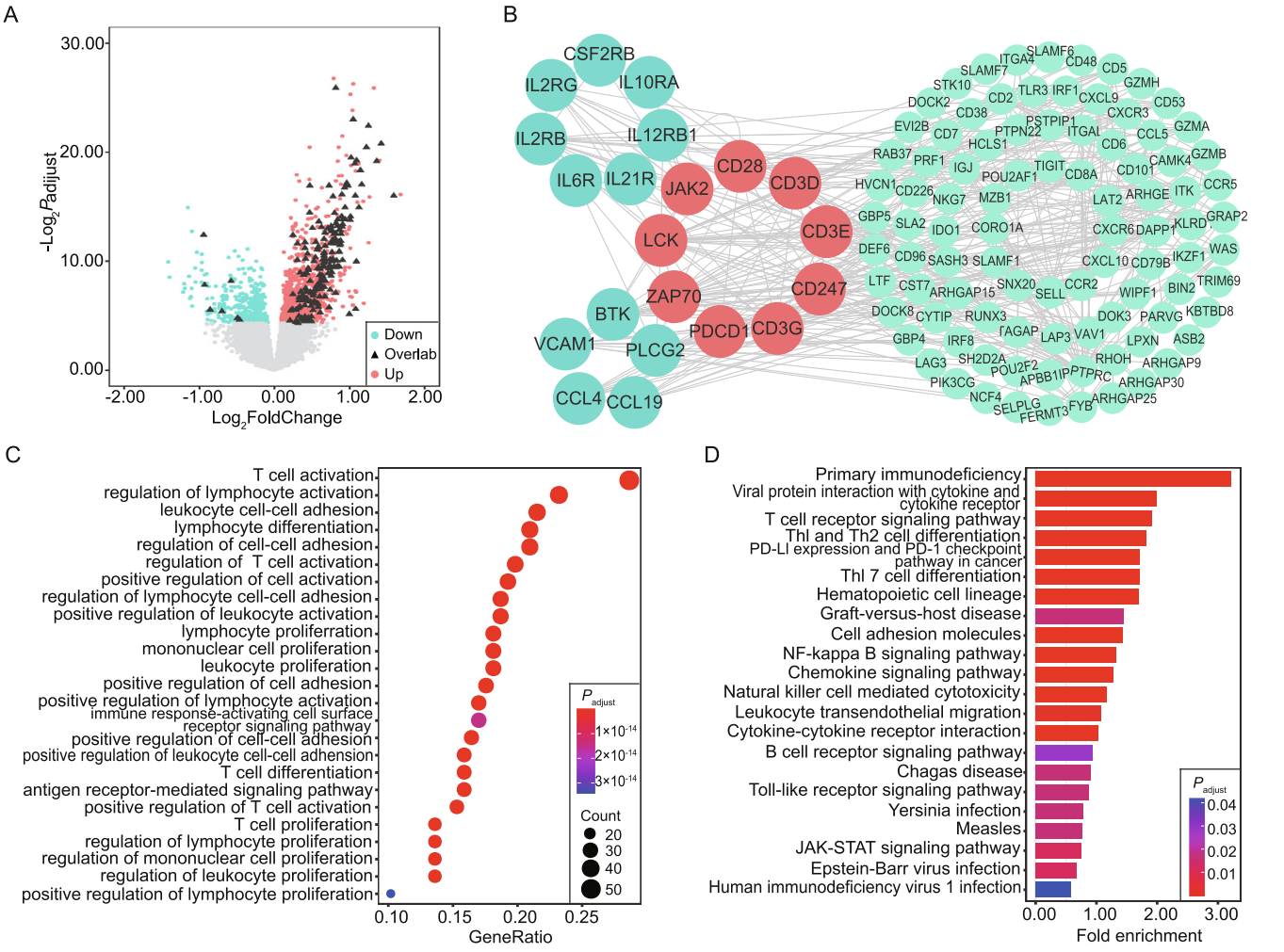


Fig. 3. Functional enrichment of differentially expressed genes identified in both Korea and THCA cohorts. (A) The volcano plot shows the 215 overlapped differentially expressed genes in the THCA cohort. (B) Protein–protein interaction network of overlapped differentially expressed genes. Gene Ontology (C) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (D) enrichment of identified differentially expressed genes.

groups of both Korea and THCA cohorts (**Supplementary Figure S4**). Indeed, the expression levels of genes related to activated T cells, including *JAK2*, *ZAP70*, *LCK*, *CD28*, *CD3D*, *CD3E*, *CD3G*, *CD247*, *PDCD1* and *BATF2*, were significantly higher in the HT groups than in the Non-HT groups in both Korea and THCA cohorts (Fig. 4**A, B**). However, beyond expectation, we observed that the expression level of the PD-1 gene (*PDCD1*) in the HT group was also significantly higher than that in the Non-HT group. Given the known immunosuppressive effects of PD-1, the high expression of the PD-1 gene usually implies the failure of the antitumor mechanism [6,8,59,61], which seems to be contradictory to this favorable prognostic value in patients with PTC concomitant with HT. However, accumulating evidence indicates that PD-1 is highly expressed on tumor-specific activated T cells [59,61,62]; thus, the high expression of the PD-1 gene further demonstrated the activation of T cells in PTC patients with concomitant HT, and it may restrain the autoimmune response of HT [63]. Overall, the results reported here highlighted the functional importance of these upregulated immune-related genes in the elimination of cancer cells in PTC patients with concurrent thyroiditis.

3.4. Single cell analysis

In view of the altered abundance of immune cells and transcriptome-wide alterations in PTC patients with Hashimoto thyroiditis, we investigated whether the differences existed between HT and Non-HT groups at the single-cell level. After quality control, we retained 17,014 genes across 17,438 cells from 6 PTC samples for subsequent analysis. We partitioned the cells into 15 major cell types with uniform manifold approximation and projection (UMAP) analysis (Fig. 5**A**), including exhausted $CD8^+$ T cells, $CD4^+$ cytotoxic T cells, plasma cells, B cells, macrophages, dendritic cells, monocytes, epithelial cells, endothelial cells, neutrophils, erythrocytes, mast cells, mesenchymal cells, smooth muscle cells and undefined cell type. By calculating the frequency of the cell types, the proportions of plasma cells, B cells, macrophage cells, exhausted $CD8^+$ T cells, and $CD4^+$ cytotoxic T cells in PTC patients with HT were higher than those in PTC patients without HT (Fig. 5**A**). As previously reported, $CD8^+$ T cells are one of the main cell types involved in thyroiditis induced by immunotherapy [7]. Hence, we performed KEGG enrichment analysis using differentially expressed genes from clusters of exhausted $CD8^+$ T cells. Excitingly, the terms "PD-L1 expression and PD-1 checkpoint pathway in cancer", "primary immunodeficiency", "T cell receptor signaling pathway" and "NF-kappa B signaling pathway" were significantly enriched (Fig. 5**B**), which further validated our aforementioned results of bulk RNA sequencing (Fig. 3**D**). Next, we focused on the expression level of PD-1-related genes in different groups (HT versus Non-HT) and observed that the expression of *CD3D* with the greatest percentage was higher in the HT group than in the Non-HT group (Fig. 5**C**). When considering

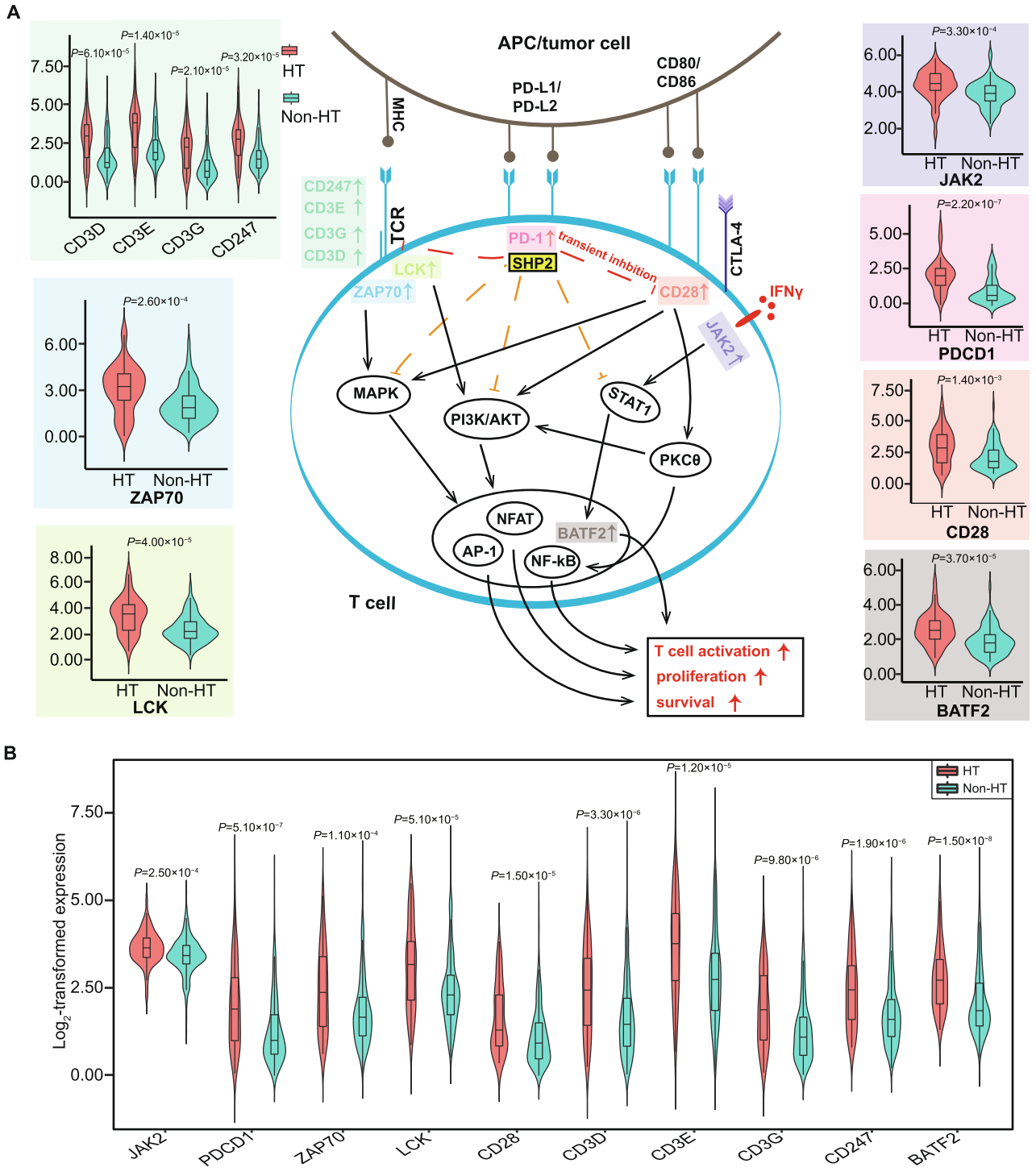
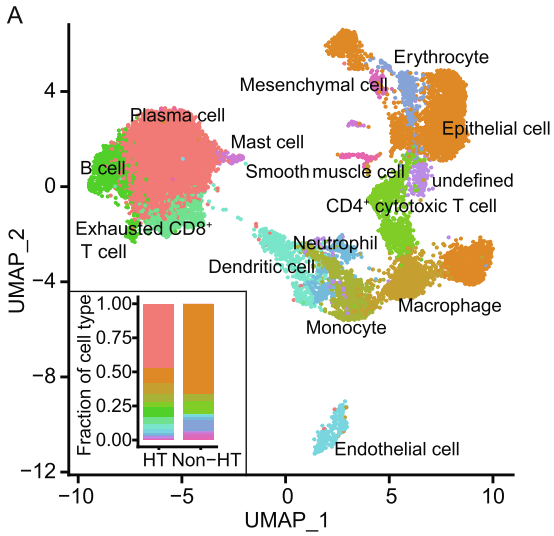


Fig. 4. PD-1-related genes differentially expressed in both Korea and THCA cohorts. (A) Simplified crosstalk between T cells and tumor cells and identified differentially expressed genes with log_2-transformed expression levels between PTC patients with HT and without HT (Non-HT) in the Korean cohort. (B) The log_2-transformed expression level of PD-1-related genes between the HT and Non-HT groups in the THCA cohort.

the expression of PD-1-related genes in different cell types, we found that *CD3D* was highly expressed in clusters of exhausted $CD8^+$ T cells and $CD4^+$ cytotoxic T cells and moderately expressed in clusters of plasma cells, B cells and mast cells (Fig. 5**D**). These results indicate that Hashimoto thyroiditis is a T cell-, especially $CD8^+$ T cell-mediated process. Taken together, these results highlight the critical role of $CD8^+$ T cells in the treatment of tumor patients, and the *CD3D* gene may act as a potential target for the activation and proliferation of $CD8^+$ T cells.

3.5. Association between thyroid dysfunction and ICI therapy

Increasing evidence shows that thyroiditis occurs frequently in cancer patients treated with PD-1 or PD-L1 inhibitors [8]. We then hope to decipher the similarity or difference between PTC patients with thyroiditis and PD-1/PD-L1 inhibitor-induced thyroiditis. After reviewing the articles about the usage of PD-1/PD-L1 inhibitors in various solid tumors, we observed that thyroid dysfunction induced by PD-1/PD-L1 inhibitors was at a rate of approximately



B

Enriched terms	Odds ratio	Pvalue	P.adjusted
Primary immunodeficiency	27.26	6.52E-8	6.98E-6
Hematopoietic cell lineage	8.72	0.00025	0.0084
Cytokine-cytokine receptor interaction	4.68	0.00025	0.0084
T cell receptor signaling pathway	8.30	0.00031	0.0084
Human immunodeficiency virus 1 infection	4.89	0.0013	0.023
Regulation of actin cytoskeleton	4.75	0.0015	0.023
Human T-cell leukemia virus 1 infection	4.67	0.0016	0.023
PD-L1 expression and PD-1 checkpoint pathway in cancer	7.76	0.0017	0.023
Th1 and Th2 cell differentiation	7.51	0.0020	0.023
Viral protein interaction with cytokine and cytokine receptor	6.90	0.0026	0.028
NF-kappa B signaling pathway	6.64	0.0030	0.029
Th17 cell differentiation	6.40	0.0034	0.029
Autoimmune thyroid disease	9.77	0.0035	0.029
TNF signaling pathway	6.16	0.0039	0.030

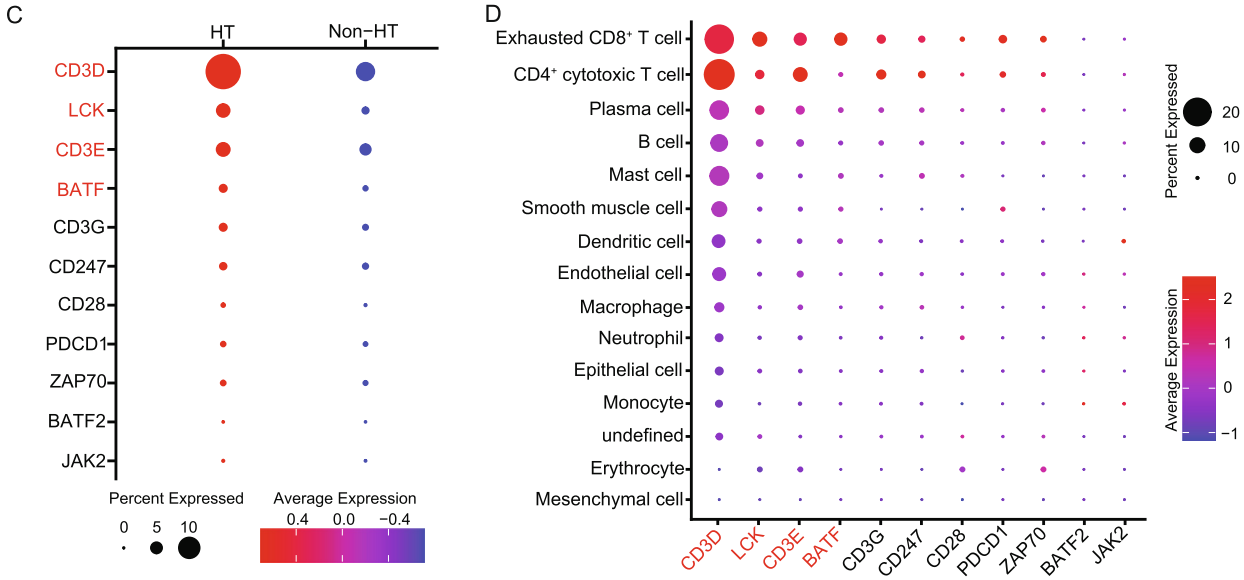


Fig. 5. Comparison of PD-1-related genes between PTC patients with HT and Non-HT at the single-cell level. (A) UMAP plots for cell type identification of 17,438 single cells from 6 PTC patients. (B) KEGG pathway analysis of differentially expressed genes from exhausted CD8$^+$ T cell clusters. Average expression of previously identified PD-1-related genes in different groups (C) and across different cell types of PTC patients with HT (D). Gene symbols colored in red indicate the genes significantly elevated in PTC patients with HT at the single-cell level.

20% [6,64–82] (Fig. 6**A**), which is consistent with previous reports [6–8]. In particular, these patients with thyroid irAEs usually had better overall survival and reduced mortality risk than those who did not [6–8]. Therefore, combined with our results, we speculate that thyroid dysfunction, especially Hashimoto thyroiditis, is a favorable biomarker for the antitumor immune response during anti-PD-1/PD-L1 immunotherapy. To confirm our hypothesis and identify potential biomarkers of thyroid irAEs in anti-PD-1/PD-L1 immunotherapy, according to Jing *et al.*'s methods [43], we retrieved a total of 3,318 thyroid adverse events from 39,123 patients across 24 tumor types from the FAERS. We calculated the odds ratio of thyroid dysfunction by comparing the proportion of reporting thyroid irAEs for anti-PD-1/PD-L1 inhibitors with that for all other inhibitors in the FAERS. We observed the highest OR of thyroid dysfunction in mesothelioma (MESO), while the lowest value was observed in uveal melanoma (UVM) (Fig. 6**B**). We collected 30 molecular signatures related to the immune therapy response, including 10 PD-1-related genes identified in our transcriptomic analysis, and further evaluated their association with thyroid dysfunction risks calculated from individual safety records of FAERS (Fig. 6**C**). Interestingly, CD8$^+$ T cells were significantly positively correlated with thyroid dysfunction OR (r = 0.72, P = 0.0031) (Fig. 6**C**), consistent with the observation that PTC patients with thyroiditis disorder had a high abundance of CD8$^+$ T cells (Fig. 2**;** Fig. 5**C**). We observed strong positive correlations between 10 PD-1-related genes and OR of thyroid irAE (i.e., CD247: r = 0.65, P = 0.0093; CD28: r = 0.64, P = 0.0093; PDCD1: r = 0.58, P = 0.02) (Fig. 6**C**). By the way, we found signature of CTLA-4, which binds to CD80/CD86 with greater affinity than CD28, resulting in CD8$^+$ T cell inactivation (Fig. 4**A**) [30], exhibited a lower correlation with thyroid irAE OR (r = 0.40, P = 0.085). We also identified several signatures, including MHC2 (r = 0.60, P = 0.017) and IFNγ (r = 0.53, P = 0.027), T cell receptors (r = 0.51, P = 0.032), B cell receptors (r = 0.54, P = 0.026) and the STAT1 score (r = 0.50, P = 0.035, that exhibited significant positive correlations with thyroid irAE OR (Fig. 6**C**), implying that the activities of these identified signatures might contribute to T cell activity and proliferation ability. Overall, these data indicate that there

A

Study	Tumor type	Drug	Total No.	Dysfunction No.	Prop (%)
Kotwal *et al.*	Lung, Prostate *et al.*	Atezolizumab,avelumab	91	23	25
Delivanis *et al.*	Melanoma, NSCLC	Pembrolizumab	93	13	14
Osorio *et al.*	NSCLC	Pembrolizumab	48	10	21
de Filette *et al.*	Melanoma	Pembrolizumab	99	17	18
Muro *et al.*	Gastric	Pembrolizumab	39	8	21
Huang *et al.*	OSCC	Camrelizumab	228	38	17
Mok *et al.*	NSCLC	Pembrolizumab	636	126	20
Eggermont *et al.*	Melanoma	Pembrolizumab	509	141	28
Toi *et al.*	NSCLC	Nivolumab	70	7	10
Cohen *et al.*	HNSC	Pembrolizumab	246	42	17
Chung *et al.*	Adenocarcinoma *et al.*	Pembrolizumab	90	20	22
Balar *et al.*	Urothelial cancer	Pembrolizumab	370	35	9
Ferris *et al.*	HNSC	Durvalumab	237	27	11
Soriano *et al.*	NSCLC	Nivolumab	35	9	26
Morris *et al.*	Anal cancer	Nivolumab	37	3	8
Hsu *et al.*	Nasopharyngeal	Pembrolizumab	27	5	19
Arrieta *et al.*	NSCLC	Pembrolizumab	40	11	28
Song *et al.*	Hodgkin lymphoma	Tislelizumab	70	23	33
Yang *et al.*	NSCLC	Pembrolizumab	12	4	33
Nghiem *et al.*	Merkel Cell Carcinoma	Pembrolizumab	50	5	10

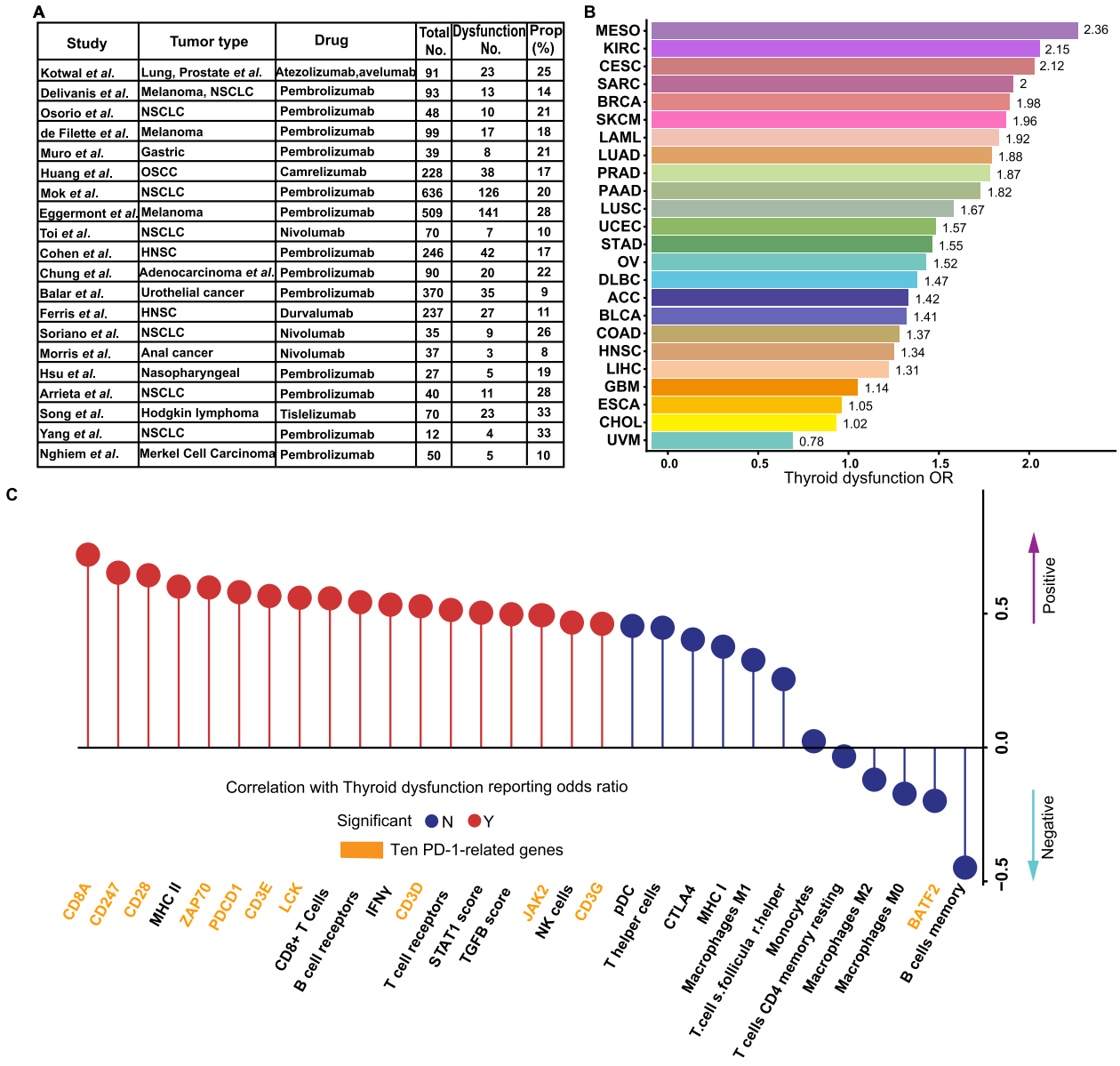


Fig. 6. Systematic evaluation of the association between immune checkpoint blockade-induced thyroid dysfunctions and molecular signatures across different cancer types. (A) Incidence of thyroid immune-related adverse events (irAEs) in patients receiving anti-PD-1/PD-L1 inhibitors. PD-L1 inhibitors include atezolizumab, avelumab and durvalumab, and PD-1 inhibitors contain nivolumab and pembrolizumab. (B) Odds ratio (OR) of thyroid irAEs by comparing the proportion of reporting thyroid irAEs for anti-PD-1/PD-L1 inhibitors with that for all other inhibitors across 24 cancer types. KIRC, kidney renal clear cell carcinoma; SKCM, skin cutaneous melanoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; BRCA, breast invasive carcinoma; MESO, mesothelioma; SARC, sarcoma; PRAD, prostate adenocarcinoma; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; UCEC, uterine corpus endometrial carcinoma; PAAD, pancreatic adenocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B cell lymphoma; OV, ovarian serous cystadenocarcinoma; HNSC, head and neck squamous cell carcinoma; LAML, acute myeloid leukemia; STAD, stomach adenocarcinoma; BLCA, bladder urothelial carcinoma; ACC, adrenocortical carcinoma; LIHC, liver hepatocellular carcinoma; GBM, glioblastoma multiforme; ESCA, esophageal carcinoma; CHOL, cholangiocarcinoma; UVM, uveal melanoma. (C) Correlation of thyroid irAE with the 30 molecular signatures. Red lollipops indicate significant correlations adjusted by false discovery rate, while blue lollipops indicate non-significant correlations.

is a similar molecular mechanism underlying thyroid dysfunction between patients receiving anti-PD-1/PD-L1 therapies and PTC patients suffering from HT.

4. Discussion

The coexistence of PTC and HT, two opposite extremes of immune responses, has been reported [17]. However, a key unanswered question in PTC combined with HT is whether and which genomic metrics are correlated with their clinical outcome. In this work, we performed clinical characteristic analysis of 468 PTC patients from two independent cohorts and meta-analysis of 22,155 PTC patients from 20 studies to determine exactly the difference in recurrence between PTC patients with (HT) and without HT (Non-HT). As a result, we observed a strong negative association between HT and recurrence in PTC patients. This result is consistent with those previously reported [18,22,52]. Thus, it is clear that, as a protective factor, Hashimoto thyroiditis improved the prognosis of recurrence in patients with PTC. Analysis of abundance of immune cells in bulk tumor tissues and single cells showed that the mean immune score and abundance of $CD8^+$ T cells in PTC patients with HT were significantly higher than those without HT (Non-HT) in both cohorts, and a high abundance of $CD8^+$ T cells was positively associated with the disease-free

survival of PTC patients. Previous reports indicated that intertumoral immune cells, especially $CD8^+$ T cells, play an essential role in antitumor immunity, and the number and function of infiltrating $CD8^+$ T cells in tumors were significantly correlated with progression-free survival [7,83]. It is reasonable to infer that the elevated abundance of these activated immune cells can lead to good prognosis of PTC patients with HT, highlighting the importance of improving the number and function of tumoral $CD8^+$ T cells during the treatment of patients who suffer from tumors. At the molecular level, increased expression of genes related to activated T cells, including *JAK2*, *ZAP70*, *LCK*, *CD28*, *CD3D*, *CD3E*, *CD3G*, *CD247*, *PDCD1* and *BATF2*, was observed in PTC patients with HT based on bulk RNA-Seq data (Fig. 4**A, B**). It should be acknowledged that we could not clearly define the source of differential expression at the cell-type level from bulk RNA-Seq data. Given that substantial variations occurred in transcriptional regulation among cell types [84], the exact contribution of cancer cells and immune cells to the observed differentially expressed transcripts needs to be delineated in the following studies. Additionally, there remain some different views regarding the relationship of the coexistence of thyroid cancer and thyroiditis [19]. Several studies have shown thyroiditis is a risk factor for the development of thyroid carcinoma [12,20–22]. Recently, McLeod *et al.* unveiled that longstanding pre-existing thyroid autoimmunity 10 years prior to thyroid cancer diagnosis was associated with risk of PTC based on a case-control study nested within the cohort of US active-duty personnel 1996–2014 [85]. Whether the molecules identified in this study contributed to the high incidence rate of carcinoma in HT patients remains to be defined in future studies.

A spectrum of endocrinopathies has been characterized during ICI immunotherapy [6]. Hypophysitis is the most frequent endocrinopathy reported with CTLA-4 inhibitor. While, thyroid dysfunction has been reported more commonly with PD-1 inhibitors, at a rate of 7–25% (Fig. 6**A**). Thus, it is imperative to examine the clinical manifestations of PD-1/PD-L1 blockade-mediated thyroid dysfunctions. This inspired us to investigate the molecular connection between ICI and induced thyroid irAEs and PTC patients with HT. In our study, differentially expressed genes between the HT and Non-HT groups in both cohorts were mainly involved in "PD-L1 expression and PD-1 checkpoint pathway in cancer". PD-1 is mainly expressed on activated T cells, which inhibit the activation of T cells by binding its ligand PD-L1 [59,61]. Beyond our expectation, we observed that the expression of PD-1 was significantly increased in the HT group compared with Non-HT group. In view of the fact that PD-1 inhibits the activity of T cells in tumor immunity, this was in contrast to our clinical observation in which the prognosis of the HT group was better than that of the Non-HT group. In our study, the HT group was considered to have activated T cells, and our above results indicate that a high abundance of $CD8^+$ T cells was beneficial to the prognosis of PTC patients. Our results were similar to the clinical results after the use of PD-1 inhibitors, both of which resulted in PD-1-induced thyroiditis. From the perspective of molecular level, T cell activation requires the interaction of the MHC with T cell receptor and several CD3 chains identified in this study, including CD3D, CD3E, CD3G and CD247 (Fig. 4**A**). It is widely believed that PD-1 would first inhibit the function of CD3-TCR complex, but hui *et al.* showed that PD-1 suppressed T cell function primarily by inactivating signaling of CD28 [86], another molecule identified in this study. Notably, it has been previously reported that both CTLA-4 and CD28 competitively bind to CD80/CD86, and CTLA-4 binds to CD80/CD86 with much stronger affinity than CD28 [87]. Our results unveiled overall upregulation of both immunostimulatory and inhibitory features in PTC patients concomitant with HT compared with PTC patients without HT. Similar characteristics have been found in some recent studies of immunotherapy, and they observed a positive correlation among inhibitory and stimulatory immune features with pathological complete response [57,88,89]. These further suggest there are similar features within the PTC patients with HT and patients who underwent immunotherapy. In this way, we proposed that the indicators of thyroid-related functions should be checked before immune checkpoint treatment, and thyroiditis can be used as an important reference for prognosis. It should be mentioned that our single-cell analysis pointed out that the *CD3D* gene was a potential target for enhancing the activation and proliferation of $CD8^+$ T cells. In this regard, it is necessary to measure the expression of *CD3D* to control the signal transduction of $CD8^+$ T cells and to avoid the overgeneration of irAEs. The findings reported here provide a basis for further exploring the utility of these biomarkers as precise cancer immunotherapy guidelines.

Our results provide a promising model for studying irAEs, and a systematic assessment of irAEs may provide important insights not only into the mechanisms that drive irAEs but also into the clinical management of the adverse events associated with cancer immunotherapy.

5. Key points

- Hashimoto thyroiditis show strong negative association with recurrence in papillary thyroid carcinoma patients, and $CD8^+$ T cells were enriched in PTC patients with thyroiditis
- Immune checkpoint-related pathway was activated in the papillary thyroid carcinoma patients with Hashimoto thyroiditis, and *CD3D* was a potential target for enhancing the activation of $CD8^+$ T cells
- Similar molecular signatures were observed between ICI therapy-induced thyroid dysfunction and thyroiditis combined with PTC

Contributors

HJT and ZSS designed and supervised the study. YL YZ, TDF, WL and QL did the bioinformatic analysis. YL, ZCL, AZL, QQW, QLL, YYL and HJT did clinical interpretation of molecular signatures. YL, HJT and ZSS wrote the manuscript. All authors read and revised the manuscript.

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Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting or dissemination plans of this research. Patient consent for publication not required.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.05.019.

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