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Original Article

Analysis of the tumor microenvironment and mutation burden identifies prognostic features in thymic epithelial tumors

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Abstract: Thymic epithelial tumors (TETs) are one of the rarest adult malignancies in the anterior mediastinum. Thymic carcinomas (TCs) are less prevalent among TETs, but they are more clinically aggressive. Immunotherapy has emerged as a promising therapeutic approach for refractory TETs, even though chemotherapy remains the conventional treatment for the advanced disease. However, limited attention has been paid to the features of the tumor microenvironment (TME) which might provide clinically relevant information and guide treatment regimen design. Especially, to date, there have been only a few studies focusing on the differences between the TME and genomic features preserved by TETs and TCs. We analyzed the TME and genomic characteristics of TETs using RNA sequencing and whole-exome sequencing, finding that distinct characteristics of TME in different pathogenic subtypes of TETs. According to those findings, we found that thymic carcinomas had significantly lower expression of HMGB1, a pro-inflammatory cytokine-related gene, than thymomas, and low HMGB1 expression was linked to a poor prognosis. Additionally, higher mutation burdens were significantly associated with the later stage and more advanced pathological types. Thymoma patients with lower mutation burdens tended to relapse within 3 years. In summary, different characteristics of TME and genomic features between thymoma and thymic carcinoma were associated with clinical outcomes of TETs and presented promisingly predictive value for efficacy and toxicity of immunotherapy.

Keywords: Thymic epithelial tumors, thymic carcinomas, thymomas, the tumor microenvironment, genomic features HMGB1

Introduction

Thymomas and thymic carcinomas (TCs) are two types of thymic epithelial tumours (TETs), and TCs are the less prevalent primary tumors in the anterior mediastinum [1, 2]. Thymomas are further subdivided into different subtypes (T-A, T-AB, T-B1, T-B2, and T-B3) based on the relative proportion of the non-tumoral lymphocytic components, and the architectures of thymomas are similar to pathologically normal thymus [3]. The 5-year survival rate of thymoma patients is approximately 90%, but that of thymic carcinomais only 55% [4, 5]. The etiology of TETs is unclear, and limited knowledge of the genomic features of thymoma and TCs was obtained.

The development of targeted therapies is thwarted by the absence of general mutations of TETs and a low incidence of the disease [6]. Immunotherapy is effective for other malignancies and may be promising in providing therapeutic opportunities for refractory TETs [7, 8].

However, the thymus has a unique role in the development of adaptive immunity, fostering the maturation of T-cells through the proper selection of non-self-reactive clones. As a result, ICIs (immune checkpoint inhibitors) treatment in TET patients is associated with a significant rate of immune-related adverse effects. Therefore, chemotherapy still represents the backbone of systemic treatment for TETs patients to date [9]. Only a single biomarker (such as PD-L1) expression is insufficient to identify TETs subtypes which show different disease-related immune characteristics and genomic features. In addition, interplay across the tumor microenvironment (TME), stromal cell components, and tumor cells establishes a dynamic connection, which affects cancer progression in terms of growth, invasion, metastasis, and immune suppression [10]. Thus, exploring the characteristics of the TME may lead to the development of prospective prognostic markers and the identification of novel therapeutic targets.

In this study, in order to deepen the understanding of the molecular underpinnings of TETs, we utilized RNA sequencing as well as whole exome sequencing (WES) to characterize different subtypes of TETs and their TME, identifying potential novel biomarkers for predicting the outcomes of TETs.

Materials and methods

Patient and samples

Surgically resected tissue samples of twenty-one patients, who were pathologically diagnosed as TETs from December 2013 to July 2017 at Sun Yat-Sen University Cancer Center (SYSUCC), were retrospectively collected. The clinical information was retrieved from the medical records. Written informed consent was obtained from all participants, and this study was approved by the institutional review board of SYSUCC.

RNA extraction

RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissues using Trizol and RNeasy MinElute Cleanup Kit (74204). RNA purity was measured using the kaiaoK5500®Spectrophotometer (Kaiao, Beijing, China). The RNA was qualified when the OD260/OD280 was between 1.8 and 2.0. RNA integrity and concentration were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). According to the manufacturer's protocol, mRNA libraries were prepared using the NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (#E7530L, NEB, USA). The RNA-seq libraries were sequenced using the HiSeq 3000 Sequencing System (Illumina, San Diego, CA, USA) with 2×101-bp paired-end reads.

DNA extraction

The tissue DNA (tDNA) was extracted from the tumors and the tumor-adjacent tissues using the QIAamp DNA MiniKit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Peripheral blood lymphocytes (PBLs) were used for the extraction of germline genomic DNA (gDNA). The concentrations of DNA were measured by Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and the Qubit dsDNA BR (Broad-Range) Assay Kit (Invitrogen, Carlsbad, CA, USA).

Gene expression profiling

The sequencing reads containing adaptor sequences and low-quality reads were removed to obtain high-quality paired-end reads. These reads were aligned to the human genome (hg19) using HISAT (v2.0.4). Transcript assembly was performed using StringTie (v1.2.3). DESeq2 was used for gene differential expression analysis [11].

Tumor-infiltrating lymphocyte subpopulations analysis

The single sample gene set enrichment analysis (ssGSEA) was used to calculate the enrichment scores (ES) of immune cell types in the tumor microenvironment [12]. The immune score and stromal score were evaluated by applying the ESTIMATE algorithm to the gene expression data [13]. Gene signatures of 28 immune cell types associated with innate and adaptive immunity were derived as previously described [14]. Tumors were further subclassified into different immune groups using the Euclidean distance and 'ward.D' clustering [15]. The tumor-infiltrating lymphocytes (TILs) enrichment profiles were constructed by pre-ranked GSEA. For each patient, expression lev-

els of genes were z-score normalized and ranked in descending order according to the z-scores. Genes associated with immune signatures were compared to the above ranked GSEA. The normalized enrichment score (NES) was obtained for each patient. We identified the immune signature gene lists with the false discovery rate (q-value) <10% and the NES >0 as enrichment.

TCR analysis

The CDR3 sequences were identified and assigned using the MiXCR software package [16]. The Shannon's entropy was calculated based on the clonal abundance of all productive TCR sequences. The normalized the Shannon's entropy (the Shannon index) was determined by dividing Shannon's entropy by the natural logarithm of the number of unique productive TCR sequences [17].

WES sequencing and analysis

A total of 1 μg DNA was fragmented into 200-250 bp segments using a Covaris S2 instrument (Woburn, MA, USA). The KAPA DNA Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA) was used to construct sequencing libraries according to the manufacturer's protocol and the libraries were hybridized to SeqCap EZ Exome 64M (Roche NimbleGen, Madison, WI, USA). In brief, the fragments were end-repaired, tailed, adapter-ligated, amplified and, hybridized to the SeqCap EZ library for 72 h, and then washed. The captured DNA was recovered using Streptavidin Dynabeads (Life Technologies), and then the captured DNA was amplified by PCR. The purified captured DNA was clustered using the cBot (Illumina, San Diego, CA, USA), and sequenced using the HiSeq 3000 Sequencing System (Illumina, San Diego, CA, USA) with 2×101-bp paired-end reads.

BWA (version 0.7.12-r1039) was employed to align the clean reads to the reference human genome (hg19). Picard (version 1.98) was used to mark PCR duplicates. Realignment and recalibration were performed using GATK (version 3.4-46-gbc02625). Single nucleotide variants (SNVs) were called using MuTect (version 1.1.4). Small insertions and deletions (Indels) were called by GATK. Somatic copy-number alterations were identified with CONTRA (v2.0.8). Mutations were considered a candidate somatic mutation only when (i) the mutation was detected in at least 5 high-quality reads containing the particular base, (ii) the mutation was not present in >1% of the population in the 1000 Genomes Project (version phase 3) or dbSNP databases (The Single Nucleotide Polymorphism Database, version dbSNP 137), and (iii) the mutation was not present in a local database of normal samples.

Statistical analysis

Comparisons of proportions and variables among different groups were performed with the Mann-Whitney U test, and paired or unpaired t-test, as appropriate. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance among multiple groups followed by Tukey's post hoc test. All statistical analyses were performed in the R statistical environment version 3.3.4 or GraphPad Prism (v. 6.0; GraphPad Software, La Jolla, CA, USA) software.

Results

Patient characteristics

A total of 21 TETs (median age at diagnosis, 45 years; male, n=14 [67%]) were enrolled in this study from Apr 2014 to July 2018. The patients' characteristics were listed in **Table 1**. There were 5, 3, 4, 3, and 6 patients in subtype T-A, T-B1, T-B2, T-B3, or TC, respectively, and all the six patients in the TC subtype had squamous cell carcinoma. The median follow-up time was 42 months, and 4 patients relapsed during this period.

Immune and stromal scores of TETs

We first evaluated the inflammation/immune profile by comparing immune and stromal scores and found that there was no significant difference in the immune score of TC compared to thymoma (P=0.1498, Mann-Whitney U test, **Figure 1A**). Further comparisons among subtypes showed that the immune scores of subtype T-A thymoma and TC were significantly lower than those of other types (P<0.001, one-way ANOVA, **Figure 1B**).

Table 1. Basic characteristics of the patient cohort

Characteristics	Total=21 (%)
Age at diagnosis	
Median (range)	45 (20-67)
Gender	
Female	7 (33%)
Male	14 (67%)
WHO Histology	
A	5 (24%)
B1	3 (14%)
B2	4 (19%)
B3	3 (14%)
Thymic carcinoma	6 (29%)
Masaoka_Stage	
I	9 (43%)
IIA	1 (5%)
IIB	1 (5%)
III	1 (5%)
IIIA	5 (24%)
IVA	2 (9%)
IVB	2 (9%)
Smoking history	
Yes	6 (29%)
No	15 (71%)
AJCC_Stage	
I	11 (52%)
II	2 (10%)
IIIA	4 (19%)
IVA	4 (19%)
Adjuvant therapy after surgery	
YES	12 (57%)
NO	9 (43%)
Family History	
NO	18 (85%)
Breast Cancer and Lung Cancer	1 (5%)
ESCC and Thymomas	1 (5%)
Liver Cancer	1 (5%)
Complications	
MG	5 (24%)
NO	16 (76%)
Recurrence	
YES	4 (19%)
NO	16 (76%)
Unknown	1 (5%)
Clinical Outcomes	
Alive	18 (85%)
Death	2 (10%)
Unknown	1 (5%)
Metastatic Sites	
NO	18 (85%)
Pleura and Lung	1 (5%)
Lung	1 (5%)
Liver	1 (5%)

Abbreviations: AJCC, American Joint Committee on Cancer; MG, myasthenia gravis.

The stromal score of TC was significantly higher than that of thymomas (P=0.0027, Mann-Whitney U test, **Figure 1C**), especially than those of T-A, T-B1, and T-B2 subtypes (P<0.01, one-way ANOVA, **Figure 1D**).

Immune infiltration of thymomas and TCs

We then applied ssGSEA on 28 immune cell signatures to assess the activity of immune cellular composition in TETs. Higher immune cell infiltration level was found in thymoma compared with TC (**Figure 2A**). We identified 14 subtypes of immune cell that had significantly different enrichment between Thymoma and TC (**Figure 2B**). High immune infiltration is contributed by cells executing anti-tumor activities (e.g., activated CD8+ T cells, activated dendritic cells, type 1 T helper cells) and cells delivering tumor suppression functions (e.g., immature dendritic cells and Myeloid-derived suppressor cells).

In addition to cellular composition in the TME, the gene expression analysis of cytokines in immune response was performed. The difference of gene expression was shown in CD40-LG, IL-10, CD40, TNFRSF14, TGFB1, MICA and HMGB1 (P<0.1, one-way ANOVA, **Figure 2C-I**; Figure S1). Among those cytokines, TNF Receptor Superfamily Member 14 (TNFRSF14) and high mobility group box 1 (HMGB1) were pro-inflammatory genes (**Figure 2F** and **2I**), but only HMGB1 showed obviously higher expression in subtypes (T-A, T-B1, and T-B2) of thymomas compared to TC (P<0.01, one-way ANOVA, **Figure 2I**). In order to examine whether the expression level of HMGB1 was associated with clinical prognosis, we investigated the TETs cohort from The Cancer Genome Atlas (TCGA). The RNA sequencing data and overall survival (OS) of 120 TETs were available for the analysis. The lower expression level of HMGB1 was associated with significantly worse OS (P=0.0245, HR=0.21, 95% CI, ranged from 0.054 to 0.81, **Figure 2J**) in this cohort.

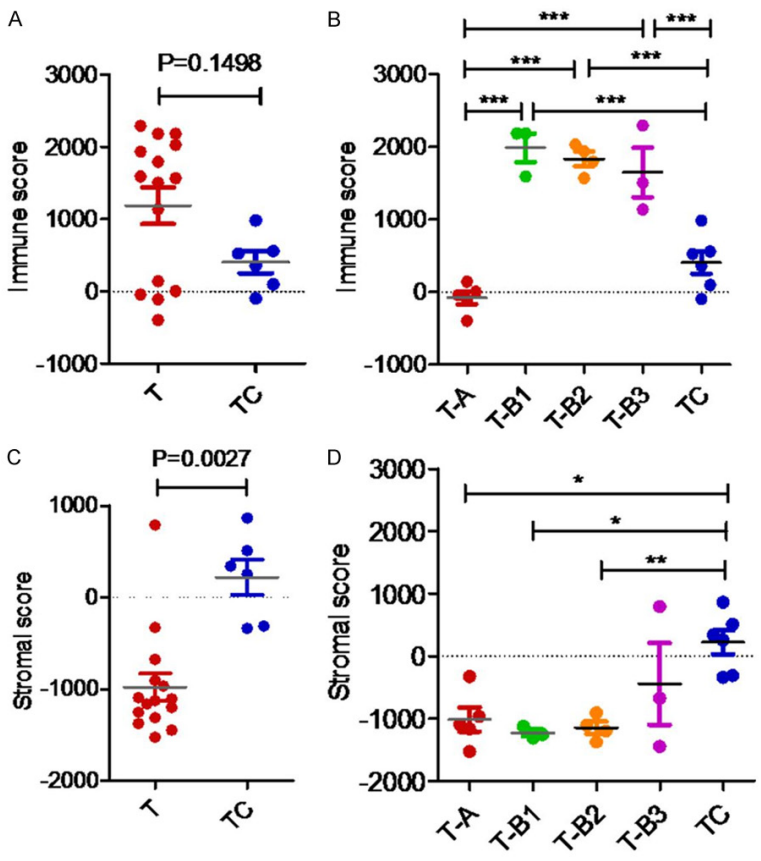

Figure 1. Immune and stromal scores of thymomas and thymic carcinomas. A. Immune scores of thymomas and thymic carcinomas (P=0.1498, Mann-Whitney U test); B. Immune scores of different subtypes of TETs (One-way ANOVA followed by Tukeys post hoc test). C. Stromal scores of thymomas and thymic carcinomas (P=0.0027, Mann-Whitney U test). D. Stromal scores of different subtypes of TETs (One-way ANOVA followed by Tukeys post hoc test). *P<0.05, **P<0.01, ***P<0.001; T: thymoma; TC: thymic carcinoma.

We further analyzed the T cell receptor (TCR) repertoire based on RNA sequencing. The Shannon index, a measure of assessing T cell diversity, was significantly higher in T-B2 and T-B3, two subtypes of thymomas, than that in TC (P<0.05, one-way ANOVA, **Figure 2K**).

Analysis of mutation burdens in thymomas and TCs

High tumor mutation burden (TMB) fosters the generation of more neoantigens, and it is capable of promoting active immune infiltration [18]. WES was performed on 15 tumors with paired tumor-adjacent tissues as germline DNA control. Genomic profiling showed that the *GTF2I* gene was mutated in all T-A patients (**Figure 3A**), consistent with previous reports. The median mutation burden of TC (44.8 mutations) was significantly higher in TC than that of thymomas (10.4 mutations, P=0.0032, Mann-Whitney U test, **Figure 3B**), and the difference of mutation burden between TC and subtypes of thymomas also showed similar tendency (**Figure 3C**). Those results suggested that neoantigens contributed partly to active immune infiltration of TETs. We next analyzed whether the mutation burden was associated with the prognosis of thymoma in the TCGA-TETs dataset and found patients who underwent recurrence within 3 years presented lower mutation burdens than those who did not (P=0.0216, Mann-Whitney U test, **Figure 3D**).

In addition, the loss of heterozygosity of HLA (HLA LOH), which has been reported to affect antigen presentation, tumor cell immune escape, and tumor response to immunotherapy in certain cancer types, was identified in 20% (3/15) of TETs, including 2 TC and 1 T-B2 patients.

Discussion

TETs are the most prevalent anterior mediastinal tumors in adults, but only a few studies have been done to offer a comprehensive review of their genetic characteristics [6, 19, 20]. Uncovering the molecular landscapes of TETs has improved our understanding of TETs oncogenesis and autoimmunity, but did not reveal targets that are actionable to currently available therapeutic agents. Although cytotoxic chemotherapies are still the standard systematic treatments for TETs, immunotherapy, especially checkpoint inhibitors that have robustly developed in recent years, presents promising efficacy and a higher rate of immune-related adverse events (irAEs) in TETs compared to other solid tumors. As a result, a detailed investigation of TETs' TME and a full understanding of immunotherapy in TETs are required.

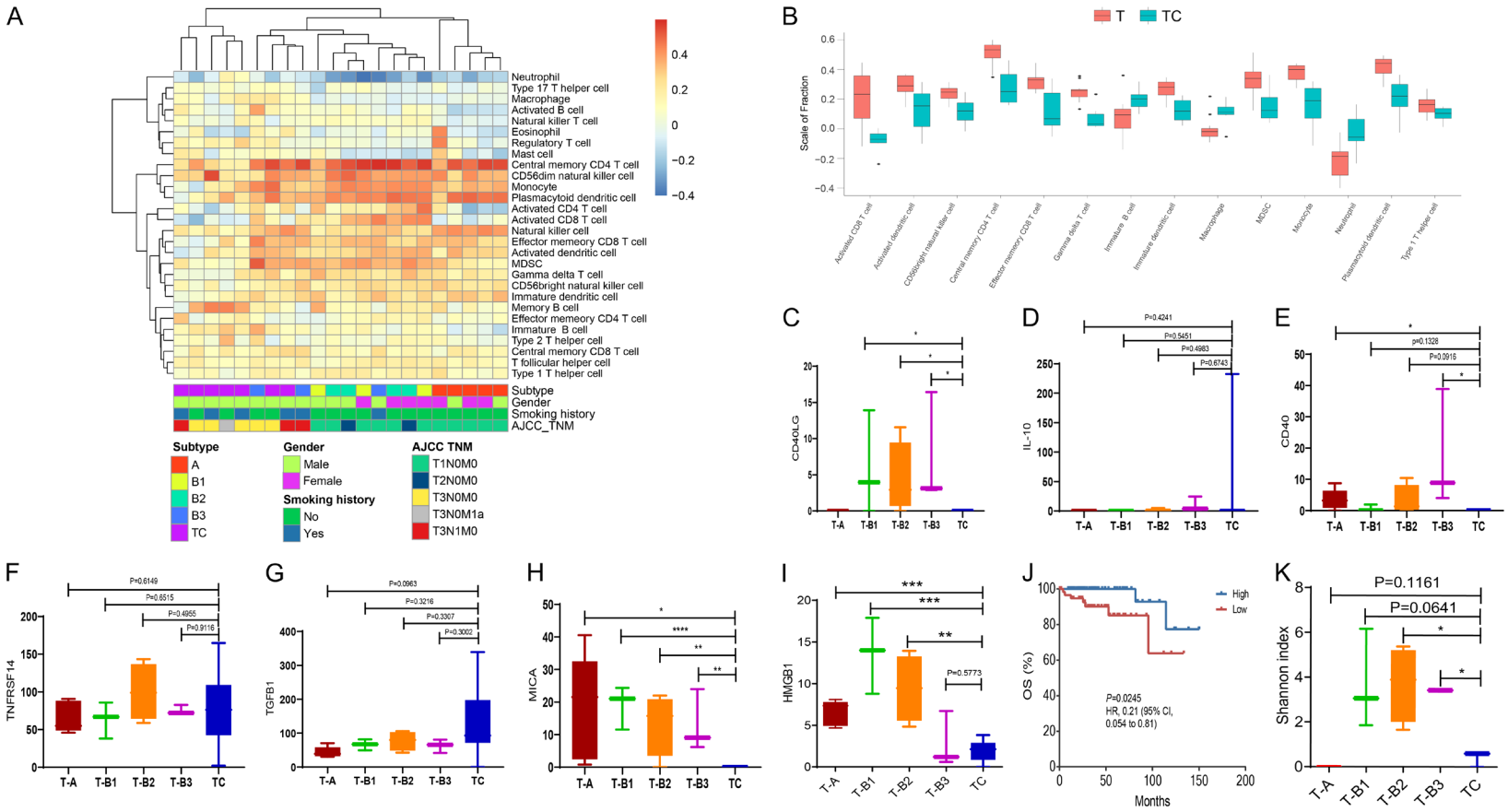

Figure 2. Immune infiltration in thymomas and thymic carcinomas. A. The classification of tumor immune infiltration by cell clusters in all samples; B. The different proportion of immune cells evaluated by ssGSEA between thymomas and thymic carcinomas (P<0.01, paired Wilcoxon test); C-H. Expression levels of cytokines related gene in immune response among different subtypes of TETs (One-way ANOVA followed by Tukeys post hoc test); I. HMGB1 expression levels among different subtypes of TETs (One-way ANOVA followed by Tukeys post hoc test); J. Lower expression levels of HMGB1 was associated with significantly worse OS (P=0.0245, HR=0.21, 95% CI, ranged from 0.054 to 0.81) in this TCGA-TETs cohort; K. T cell receptor (TCR) repertoire analysis showed Shannon index among different subtypes of TETs (One-way ANOVA followed by Tukeys post hoc test). *P<0.05, **P<0.01, ***P<0.001; T: thymoma; TC: thymic carcinoma.

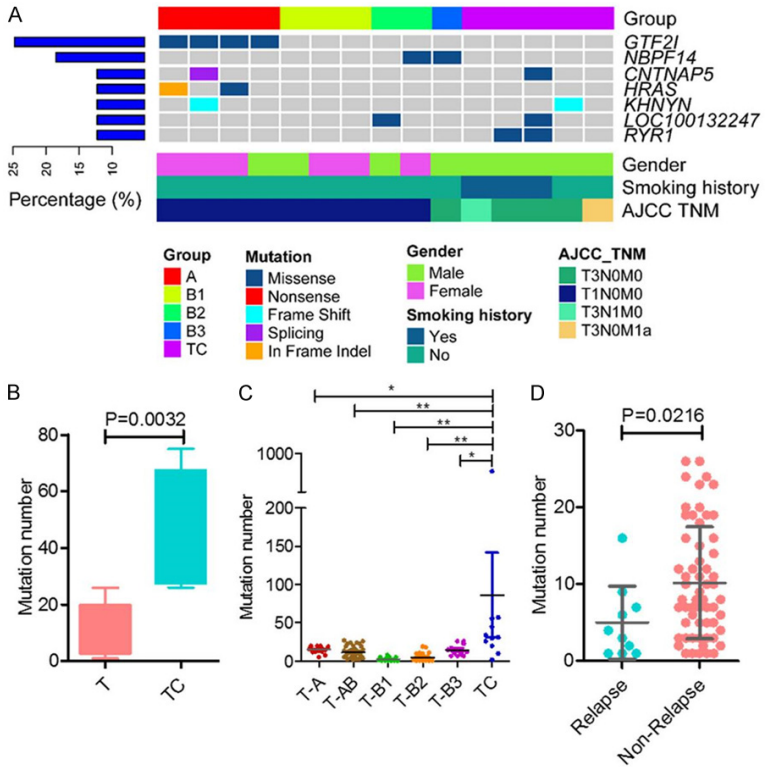

Figure 3. Genomic features and mutation burdens of TET patients. A. Somatic mutation profile in TETs; B. Significantly higher mutation burden of thymic carcinomas than that of thymomas; C. Mutation burdens of different subtypes of thymic epithelial tumors. One-way ANOVA followed by Tukeys post hoc test was applied (*P<0.05, **P<0.01); D. In thymomas cohort of TCGA-TETs, patients who suffered recurrent disease within 3 years presented lower mutation burden than those without relapse in 3 years.

In this study, we found that different constitutes of the TME across different subtypes of TETs: higher immune score (statistical insignificance may owe to small sample size), higher immune cell infiltration level, and T cell diversity in thymoma, higher stromal score, significantly lower expression of HMGB1 (a pro-inflammatory cytokine related gene), which was associated with poor prognosis, and higher mutation burden in TC. To our knowledge, our study, for the first time, utilized RNA sequencing to show the variant patterns of the microenvironment among different pathological subtypes.

The immune and stromal scores were derived from the immune components and defined by immune cell infiltration based on ssGSEA. Several studies have proved that higher immune/stromal scores are associated with a favorable prognosis of human malignancies, such as hepatocellular carcinoma, pancreatic cancer, melanoma, and lung cancer [21]. In this study, a lower level of the immune score was found in TC than in thymoma, which suggested that insufficient immune infiltration of TC might lead to failures in tumor immune surveillance and trigger tumor immune escape. However, different from observations in other malignancies, stromal scores in subtypes of TETs were inconsistent with the immune scores, anda lower level of stromal score found in thymoma than TC. The underlying reason still needs further investigation, and this finding also suggests that the thymus as an immune organ possesses more complicated TME, especially the immune environment, and more complex factors might need to be considered in the application of ICIs in TETs.

Our study found a lower expression level of *HMGB1* in TC compared to T-A, T-B2 and T-B3, three subtypes of thymoma. *HMGB1* is passively released by damaged or necrotic cells, serving as a damage-associated molecular that alerts and activates the innate immune system [22]. *HMGB1* is also secreted by activated immune cells and endothelial cells, giving rise to the release of additional pro-inflammatory cytokines and chemokines [23]. The ability of *HMGB1* to respond to cellular stress signals and sustain long-term inflammation may inhibit tumorigenesis and progression [24]. We also found that a lower expression level of *HMGB1* was associated with worse OS in the TCGA-TETs cohort, suggesting its potential prognostic value in this cancer type.

In our research, a lower mutation burden was linked to a higher probability of recurrence in TETs. Given that certain mutations can result in the production of neoantigens, the prognostic effect of mutation burdens in TETs may, in part, be attributed to the role of mutations in shaping tumor-triggered immune interactions. A similar association of high TMB (>8 mutations/

Mb) with favorable outcomes (i.e., overall survival, disease-free survival) was also reported previously in other malignancies, such as resected NSCLC [25].

Lack of actionable mutations constitutes the main reason for the paucity of developing effective targeted therapies in TETs, but high expression of PD-L1 on tumor cells and abundant CD8+ lymphocytes provide a strong rationale for implementing ICIs which target the PD-1/PD-L1 pathway in the treatment of TETs. Two phase II trials evaluated the activity of pembrolizumab (PD-1 inhibitor) in patients with recurrent TETs, Giaccone et al. included thymic carcinoma and reported a response rate of 22.5%, Cho et al. included thymoma and thymic carcinoma, and reported response rates of 28.6% and 19.2% respectively, and the incidence of irAEs was higher in thymoma than in TC (71.4% v 15.4%, respectively) [7, 8]. Both trials identified an association of high PD-L1 expression with better tumor response but failed to correlate PD-L1 expression with irAEs. Conflicting results were observed previously regarding the association of high expression of PD-L1 and histology (thymoma or thymic carcinoma), as well as survival prognosis [26-30]. TMB, microsatellite instability [31], and deficient DNA mismatch repair (dMMR) have been applied in other solid tumors [32-34], but the efficacy of those biomarkers in predicting the efficacy of immunotherapy in TETs remains debatable, not to mention their predictive value in monitoring the risk of irAEs. Based on the above, other biomarkers associated with efficacy and autoimmune-related toxicity of immunotherapy should be explored. Our study provided the distinct patterns of the tumor microenvironment among different subtypes of TETs, and revealed the level of tumor immune infiltration, TCR expression profile, the expression level of inflammation-related genes, and HLA LOH. Most of the above were found differently in thymoma and thymic carcinoma, suggesting their potential as a predictive biomarker for efficacy and toxicity risk of immunotherapy, and worth further investigation in future clinical trials. Notably, consistent with the previous report [35], *GTF2I* gene mutation was found in all T-A patients in our current study, which was also identified in a patient developing a severe autoimmune disorder in the pembrolizumab phase II trial. Along with the diverse tumor microenvironment in thymomas revealed in the study, we suggest ICIs should be administered with caution in thymomas.

Despite the long follow-up time in our study, our conclusions may be affected by the single-center retrospective analysis and relatively modest sample size. Additional prospective studies which enroll TETs patients receiving ICIs and characterize their basic multi-omics features are urgently required. Furthermore, single-cell RNA sequencing could explore those architectures of infiltrated immune cell subsets with higher resolution and construct a more detailed immune atlas of TETs.

In conclusion, those findings demonstrated that distinct pathogenic subtypes of TETs have varied TME compositions. TME and genetic differences between thymoma and TC were linked to differences in TETs prognosis, and more studies are needed to see if they may be used as a predictive biomarker for ICIs' efficacy and toxicity.

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Disclosure of conflict of interest

None.

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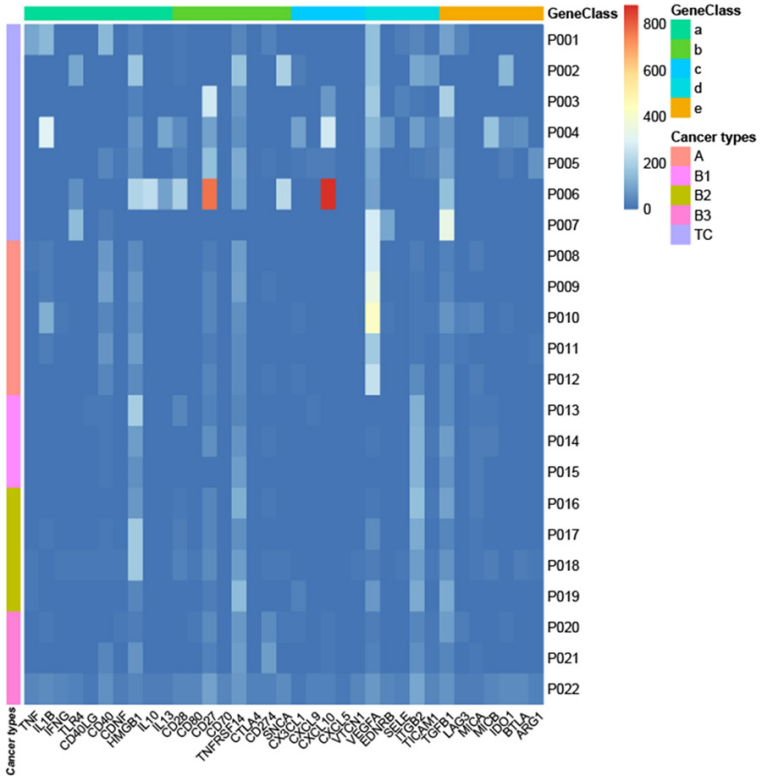

Figure S1. Heatmap showing the expression of genes involved in the immune response process in different subtypes of TETs. A: thymoma A, B1: thymoma B1, B2: thymoma B2, B3: thymoma B3, TC: thymic carcinoma, a: cancer-antigen presentation, b: priming and activation, c: trafficking of T cell to tumors, d: infiltration of cells into tumors (CTLs, endothelial cells), e: killing of cancer cells.