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Somatic Mutations of Thymic Epithelial Tumors Identified in the Prospective THYMOGENE Trial

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ABSTRACT

Background: The molecular landscape of thymic epithelial tumors has been partially elucidated. GTF2I mutation drives the pathogenesis in approximately 50% of tumors; however, the key molecular aberrations in the other cases remain unclear.

Methods: We designed a panel including the most frequently mutated genes in thymic epithelial tumors and sequenced tumor and normal DNA from 70 patients prospectively accrued at a single institution in the Thymogene trial. Moreover, 19 neoplastic samples were dissociated to isolate tumor cells using flow cytometry.

Results: GTF2I mutations were the most common, being present in 41% of patients. GTF2I mutations were prevalent in type A and AB thymomas, in Stage I–II tumors, and in patients without myasthenia gravis. The unique pattern of mutually exclusive and co-occurring mutations suggests a distinct pathogenesis for thymomas with and without GTF2I mutation. In 39% of patients, no mutations were found in the 77 genes evaluated. The absence of epithelial cells in some dissociated tumors highlights the challenge of identifying mutations in a subset of thymic epithelial tumors that lack the GTF2I mutation. Mutational signatures, including COSMIC 1, 19, and 25, were enriched, possibly linked to 5′-methylcytosine deamination and the effects of chemotherapy.

Conclusions: GTF2I mutations drive the growth of a significant portion of thymic epithelial tumors, often in conjunction with other gene mutations. Somatic mutations are not commonly found in many GTF2I wild-type tumors, where the underlying genomic abnormalities remain elusive, even when using a dedicated tool for sequencing thymic epithelial tumors.

1 | Introduction

Thymic epithelial tumors (TETs) are rare neoplasms of the anterior mediastinum [1]. TETs are classified into thymic carcinomas and thymomas, which are further subdivided into histotypes A, AB, B1, B2, and B3. Mixed histotypes, including B1–B2 and B2–B3, are common, and the 2021 WHO classification has introduced the concepts of metaplastic thymoma and micronodular thymoma with lymphoid stroma [1]. While thymic carcinomas are notably aggressive tumors, thymomas can exhibit variable behavior, typically more indolent in histotypes A, AB, and B1 and more assertive in B2 and B3 [1]. Myasthenia Gravis is the most common immune-mediated paraneoplastic syndrome observed in up to 40% of thymomas, but it is absent in thymic carcinomas [1]. Tumor stage and

completeness of resection are the main prognostic factors. Surgery is curative for localized tumors and palliative for metastatic thymomas [2]. In contrast, chemotherapy remains the standard treatment for metastatic TETs, but with limited efficacy, highlighting the need for more effective systemic therapies [2, 3]. Recently, immunotherapy and anti-angiogenic drugs, including ramucirumab, lenvatinib, and sunitinib, have shown promising efficacy in thymic carcinomas; however, they are not curative [4–7]. Currently, molecular biomarkers for targeted sequencing remain elusive in TETs, despite several attempts to define the pattern of somatic mutations in these tumors [8–12]. Therefore, a deeper understanding of somatic mutations in TETs could reveal novel therapeutic options. While GTF2I mutations are the most frequent in A and AB thymomas [8, 9], thymic carcinomas often harbor mutations in CDKN2A, TP53, and CDKN2B [13]. Limits exist in the definition of the mutational landscape of TETs, including the presence of nonneoplastic thymocytes that dilute tumoral DNA, reducing the sensitivity of the sequencing. Moreover, a peculiar pattern of mutations is observed in thymomas. For example, GTF2I mutations are not found in other types of tumors, which limits the diagnostic performance of sequencing panels that include genes commonly mutated in different types of tumors. Therefore, we collected literature data reporting somatic mutations in TETs to develop a dedicated panel of genes frequently mutated in TETs [10]. By limiting our sequencing target only to genes commonly mutated in TETs, we achieved a deep coverage, enabling the identification of mutations, even in tumors rich in nonneoplastic thymocytes. We previously sequenced only the tumor DNA of 67 thymomas with myasthenia gravis [10]; herein, we evaluated a completely independent series of 70 cases, for which we prospectively collected tumor and germline DNA within the Thymogene trial conducted at a single institution.

2 | Materials and Methods

2.1 | Patients' Selection

A consecutive series of 70 TETs was prospectively collected between January 2021 and August 2023 at the Thoracic Surgery and Robotic Surgery Units of the University Hospital of Pisa. The ethical committee of the Northwest Area of the Tuscany Region approved the Thymogene trial (CEAVNO: 18027/2020), and all patients signed the informed consent. Tumor samples, obtained during surgical resections, were immediately frozen at −80°C. Moreover, 19 tumors were also processed for cell dissociation. Five mL of blood was collected in EDTA tubes for germline DNA extraction from each patient. The staging was defined according to the Masaoka–Koga system [14], histology was classified according to the 2021 WHO guidelines [1], and myasthenia severity was assessed using the Myasthenia Gravis Foundation of America MGFA classification [15].

2.2 | Nucleic Acid Extraction

Simultaneous extraction of tumor DNA and RNA was performed using the AllPrep DNA/RNA kit (Qiagen, Hilden, Germany) following the vendor's protocol. Germline DNA was extracted from blood using the QIAamp DNA Blood Mini kit (Qiagen) following the vendor's instructions.

2.3 | Targeted Resequencing

In our laboratory, we designed a dedicated assay for sequencing by selecting the most frequently mutated genes in TETs. We reviewed the literature reporting NGS results of TET and identified 4208 mutations among 339 patients. The 63 most mutated genes were selected and included in a panel of 77 genes, along with genes frequently mutated in other tumors, as an internal control, as previously described [10]. The custom panel was projected using Design Studio for the DNA prep with the Enrichment kit (Illumina, San Diego, California). Paired-end reads of 101 nucleotides of tumor and blood DNA were generated using a NextSeq 500/550 Mid Output Kit v2.5 for NextSeq550 (Illumina).

2.4 | Bioinformatic Analysis of NGS

FASTQ files were demultiplexed, adaptors were trimmed, and barcode sequences were removed. Using the Dragen Enrichment pipeline on BaseSpace Sequence Hub (Illumina), reads were aligned to the GRCh37/hg19 reference genome in BAM files. Somatic mutations were identified using the Dragen Somatic pipeline with Tumor-Normal matching and reported in the Somatic Variant Calling Format (VCF) file. Somatic mutations detected with Vardict [16] were compared to Dragen results. Only coding and splicing site mutations that passed Dragen's filtering were included, requiring at least 50 reads of coverage. In tumor DNA, mutations with over five altered reads were selected, while those with an allele frequency above 90% were excluded as likely homozygous polymorphisms. Mutations with an allele frequency above 1% in Blood DNA were excluded. Somatic mutations were manually curated using IGV [17], and those found in germline DNA across different samples were excluded as potential sequencing errors. Finally, mutations were annotated with Annovar [18] and analyzed using the R package MAFtools [19]

2.5 | Enzymatic and Mechanical Digestion of the Fresh Biopsies

Tumor biopsies were minced and placed in a C-tube (Miltenyi Biotec, Bergisch Gladbach, Germany) with RPMI 1640 medium (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) supplemented with 2% Fetal Bovine Serum (Gibco) and an enzyme cocktail for digestion: Dispase (Roche) 200 μg/mL; DNase (Roche) 500 μg/mL; Collagenase Type 4 (Roche) 200 μg/mL. Mechanical digestion was performed using gentleMACSTM Dissociators (Miltenyi Biotec). The samples were then incubated on an Eppendorf ThermoMixer C thermoblock for 45 min at 37°C at 350 RPM and then filtered using a MACS SmartStrainer 100 μm (Miltenyi Biotec).

2.6 | Flow Cytometry

Flow cytometry was performed using the MACSQUANT Analyzer 10 Flow Cytometer (Miltenyi Biotec), employing 7-AAD, CD3-VioBlue, CD45-APC, and CD326-PE (Miltenyi Biotec). The data were analyzed using MACSQuantify Software (Miltenyi Biotec).

Gating strategy is shown in Figure 2B–F: after exclusion of cell debris on FSC (forward scatter) versus SSC (side scatter) density plots (Figure 2B), and doublets on FSC-A versus FSC-H (Figure 2C), 7-AAD staining was used to assess cell viability, and 7-AAD–negative cells were selected (Figure 2D). The obtained viable singlets were distinguished into CD45- CD3- cells (Figure 2E) and subsequently evaluated for CD326 (EPCAM) expression. Thymic epithelial cells were considered CD45- CD3- CD326+ (Figure 2F).

3 | Results

3.1 | Patients

Surgery was performed for 58 patients with primary tumors and 12 with pleural metastases. Seven patients received chemotherapy before resection. Patients' characteristics are reported in Tables 1 and S1. A correlation was found between WHO histotypes and stage. A, AB, and B1 thymomas, exhibiting a more indolent behavior, were more frequently diagnosed in Stages I–II (40%) compared to Stages III–IV (4%). Conversely, B1/B2, B2, B2/B3, and B3 thymomas and thymic carcinomas were more frequently diagnosed in Stages III–IV (31%) than Stage I–II (25%; chi-squared $p = 0.0001$). Resection of relapses was more commonly performed in more aggressive (92%) compared to more indolent histotypes (8%; Fisher's exact test, $p = 0.009$). Myasthenia gravis was more common in B thymomas (50%) than in A–AB histotypes (10%; chi-squared $p = 0.0112$).

TABLE 1 | Patients' characteristics.

Parameters	Number of patients	(%)
Age	Median 58	Range (25–82)
Sex	M 29	41%
	F 41	59%
WHO histotype		
A	3	4%
AB	21	30%
B1	6	9%
B1/B2	4	6%
B2	11	16%
B2/B3	11	16%
B3	6	9%
TC	6	9%
MNT	1	1%
MPT	1	1%
Stage MK		
I	8	11%
IIA	17	24%
IIB	21	30%
III	11	16%
IV-A	12	17%
IV-B	1	1%
Primary	58	83%
Relapse	12	17%
Myasthenia gravis	34	49%
Preoperative chemotherapy	7	10%

Note: Primary/relapse indicate if the sample used for sequencing has been collected during surgery for primary tumor or relapse.
Abbreviations: MNT, micronodular thymoma; MPT, metaplastic thymoma; stage MK, stage according to Masaoka and Koga defined at the first diagnosis of thymic epithelial tumors; T, thymoma; TC, thymic carcinoma.

3.2 | Sequencing Results

The mean coverage depth was 768 (SD ±177) and 853 reads (SD ±130) for tumor and normal DNA, respectively. The mean percentage of the Browser Extensible Data (BED) covered with at least 50 reads was 97% (SD ±12) for tumor and 98% (SD ±0) for blood. Sequencing metrics are summarized in Tables S2 and S3.

A total of 150 mutations were identified: 84 missense, 54 synonymous, five nonsense mutations, one frameshift insertion, and six frameshift deletions (Figure 1A, Table S4). The distribution of single-nucleotide mutations in each sample is depicted in the box plot of Figure 1B, indicating that several tumors exhibit only one adenine-to-thymine transversion.

Enriched signatures of mutations include COSMIC 25, possibly related to the effects of chemotherapy, COSMIC 19 of unknown etiology, and COSMIC 1 related to the spontaneous deamination of 5′-methylcytosine (Figure 1C). There were 27 TETs without evidence of mutations, 23 with only one mutation, and 20 with more than one mutation. A B2 thymoma had the highest number of mutations ($n = 24$). In this series, mutations were more common in thymomas than in thymic carcinomas, with an average of 2.19 and 1.67 mutations per sample, respectively, but the difference was not significant (T-test $p = 0.784$; Figure 1D). The most mutated genes were GTF2I (29 patients), HRAS, and SETD2 (five patients each; Figure 1E and Tables S5 and S6). GTF2I mutations consistently resulted in the missense substitution of a leucine with a histidine. Thymic epithelial cells express beta and delta isoforms of GTF2I, and the mutation can lead to the L404H and L383H missense substitutions, respectively [8]. GTF2I mutation was observed in all types of TETs except for the single case of metaplastic thymoma. A and AB thymomas more commonly present GTF2I mutations (75%, chi-squared p-value = 0.01; Table 2). TETs with GTF2I mutation have a more favorable prognosis [8]. Indeed, the mutation was more frequent in tumors diagnosed at earlier stages (Stage I–II) and in primary tumor resection, being absent in samples obtained from metastases. Interestingly, the GTF2I mutation was more common in tumors without myasthenia gravis.

The table of interactions demonstrates an intricate network of 63 significantly co-occurring mutations without mutually

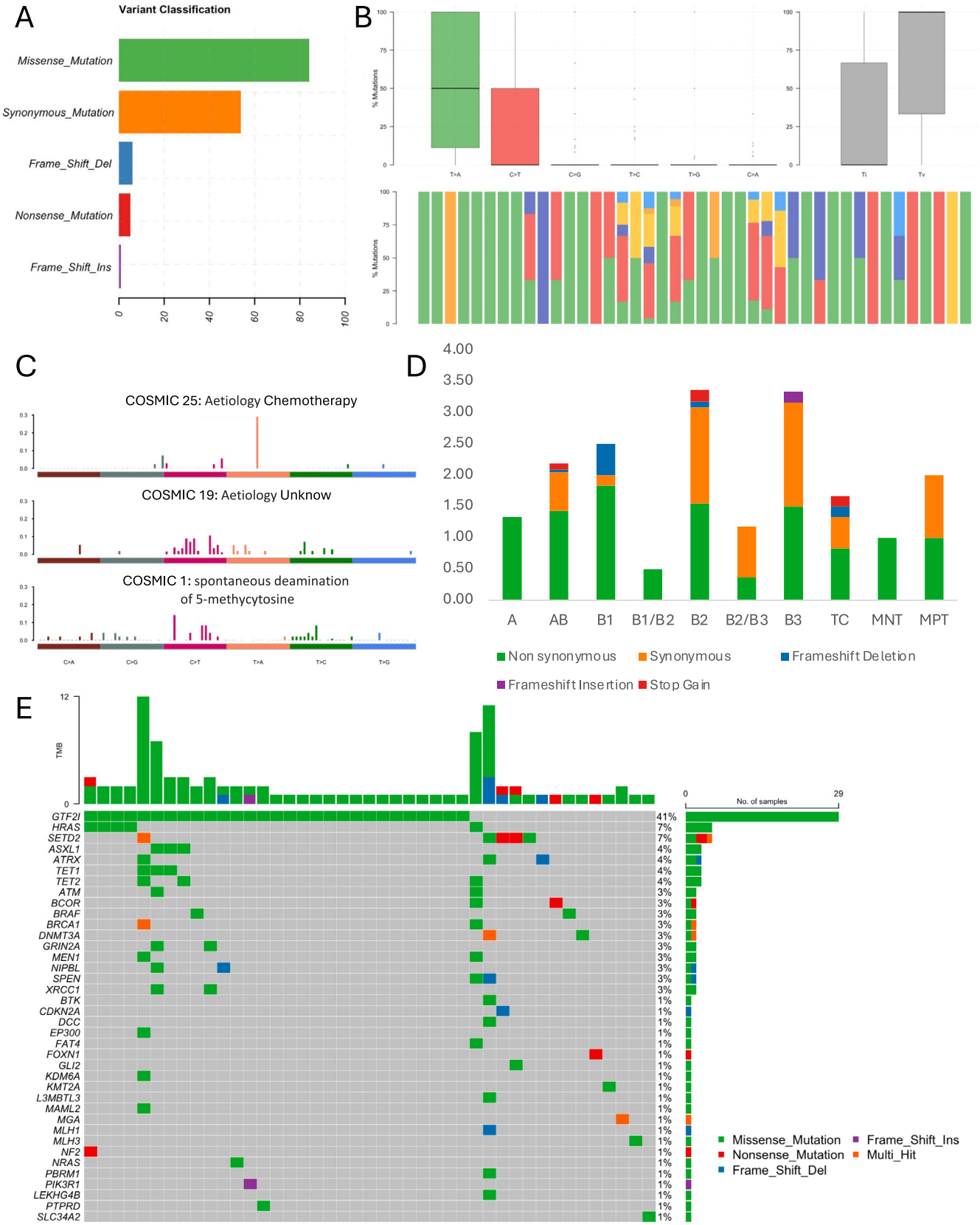

FIGURE 1 | Legend on next page.

exclusive events, suggesting a multistep process of carcinogenesis. However, no considerably co-occurring or mutually exclusive mutations with those of GTF2I were observed (Figure 2A). However, four out of five HRAS mutations and all three mutations of ASXL1 and TET1 occurred together with those of GTF2I. On the contrary, four out of five mutations of SETD2

FIGURE 1 | Characteristics of the observed mutations: (A) The number of mutations categorized by their exonic function: Missense, synonymous, nonsense, frameshift deletion, and frameshift insertion. (B) A box plot illustrating the overall distribution of six different nucleotide substitutions, a box plot of transition/transversion, and a representation depicting the fraction of substitution in each sample. (C) Enriched COSMIC signature of mutations in TETs. (D) The average number of mutations in each thymic epithelial tumor histotype, categorized by their exonic function: Missense, synonymous, nonsense, and frameshift. (E) Oncoplot displaying the most frequently mutated genes in TETs and the type of mutation, excluding synonymous mutations. This figure summarizes the results from 43 out of 70 samples with at least one mutation.

TABLE 2 | GTF2I according to patients' characteristics.

Parameters	Number of patients	GTF2I mut	GTF2I wt	
Age		Median 63 (25–80)	Median 57 (33–82)	$p = 0.92$
Sex				$p = 0.99$
M	29	12	17	
F	41	17	24	
WHO histotype		(A, AB, vs. others)		$p = 0.001$
A	3	2	1	
AB	21	16	5	
B1	6	1	5	
B1/B2	4	2	2	
B2	11	3	8	
B2/B3	11	1	10	
B3	6	1	5	
TC	6	2	4	
MNT	1	1	0	
MPT	1	0	1	
Stage MK*		(I, II vs. III, IV)		$p = 0.0115$
I	8	4	4	
IIA	17	12	5	
IIB	21	8	13	
III	11	4	7	
IV-A	12	1	11	
IV-B	1	0	1	
				$p = 0.0009$
Primary	58	29	29	
Relapse	12	0	12	
Myasthenia gravis				$p = 0.047$
Yes	34	10	24	
No	36	19	17	
Preoperative chemotherapy				$p = 0.691$
Yes	7	2	5	
No	63	27	36	

Note: The statistical significance values were calculated using Fisher's exact test or the chi-squared test when appropriate; the *T*-test was used for the age difference.
Abbreviations: MPT, metaplastic thymoma; MK, Masaoka and Koga; mut, mutation; TC, thymic carcinomas; TMN, microndular thymoma; wt, wild type.
*Indicates a statistically significant co-occurring or mutually exclusive mutation.

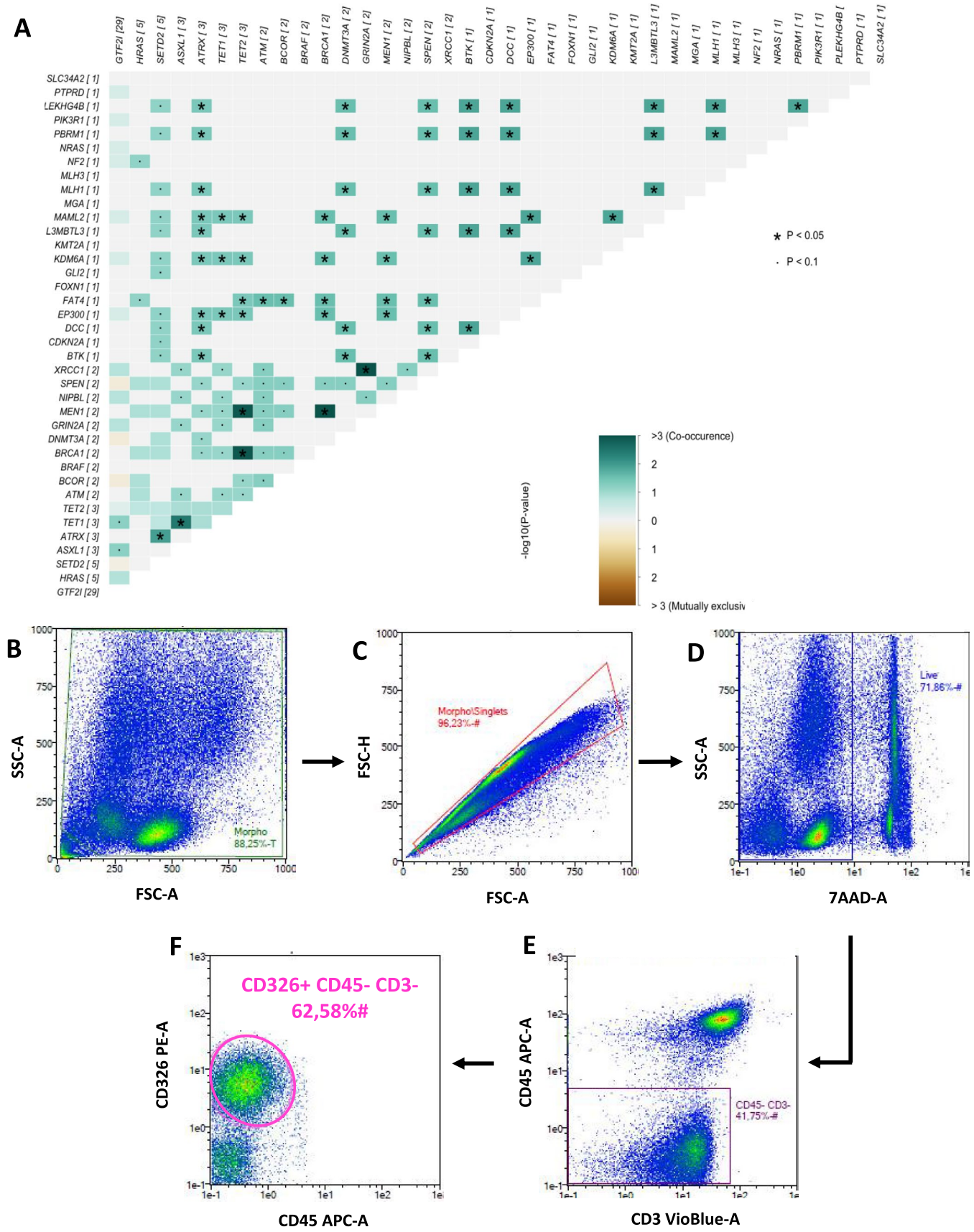

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FIGURE 2 | (A) Concordance table of co-occurring or mutually exclusive mutations. Significant co-occurrence was highlighted with "*" if $p < 0.05$ and with "•" if borderline significant with $p < 0.1$. (B–F) Graphic representation of the gating strategy on one sample of thymic epithelial tumor. Data generated by flow cytometry were visualized through density plots. (B) Cell debris and aggregates were excluded on FSC-A versus SSC-A plots. (C) Then, doublets were excluded on FSC-A versus FSC-H plots. (D) Using 7-AAD, only viable cells were selected (7-AAD negative staining). (E) The obtained singlets were further distinguished into CD45-CD3- cells. (F) CD45-CD3- cells were then selected in the CD326+ plot. The total percentage of CD45- CD3- CD326+ represents the thymic epithelial cell population. In the presented case, the thymic epithelial tumor cells were 62.58%.

TABLE 3 | Quantification of epithelial cells using flow cytometry of dissociated tumor samples.

SAMPLE	WHO	GTF2I	MUTATIONS	% EPCAM
TET-13	B2/B3	WT	NO	1
TET-29	B2	WT	NO	0
TET-50	B2/B3	WT	NO	1
TET-53	B1	WT	YES	0
TET-63	AB	WT	YES	28
TET-69	B1	WT	YES	0
TET-77	B2	WT	NO	0
TET-30	B2/B3	WT	NO	0.5
TET-38	B3	WT	YES	0
TET-51	B2/B3	WT	NO	1
TET-62	B1/B2	WT	NO	0
TET-17	AB	MUT	YES	3
TET-43	TMN	MUT	YES	1.8
TET-32	AB	MUT	YES	2
TET-58	AB	MUT	YES	8.8
TET-74	AB	MUT	YES	0.5
TET-56	B1	MUT	YES	2.6
TET-16	AB	MUT	YES	4
TET-25	B1/B2	MUT	YES	0.1

Note: WHO indicates thymoma histotype; GTF2I indicates the presence of the mutation (MUT) or wild type (WT) tumors; %GTF2I indicates the allele frequency of GTF2I mutations according to the sequencing data; %EPCAM indicates the percentage of EPCAM-positive CD45-negative cells evaluated using flow cytometry after mechanical and enzymatic dissociation of the tumor samples.
Abbreviation: TMN, micronodular thymoma.

and all two mutations of BCOR and DNMT3A were mutually exclusive with those of GTF2I.

3.3 | Correlation Between GTF2I Mutation and the Percentage of Epithelial Cells

Tumor dissociation was performed on 19 samples with sufficient material available. Flow cytometry was used to quantify the percentage of thymic epithelial cells using the gating strategy described in the Methods section. An example of the gating strategy on one sample of TET is illustrated in Figures 2B–F, in which the percentage of TEC was 62.6% of the CD45-CD3- cells. The rate of epithelial cells detected in each sample was lower than expected and is reported in Table 3. The average number of epithelial cells of the 19 samples tested was 4.5 (SD ±0.5) and 2.9 (SD ±7.9) in tumors with at least one or without mutations (*T*-test *p*-value = 0.170). The average number of epithelial cells was 2.8 (SD ±2.7) and 2.9 (SD ±8.3) in GTF2I-mutated and wild-type tumors (*T*-test *p*-value = 0.996). We did not encounter any samples with GTF2I mutations that lacked detectable tumor cells via flow cytometry (Fisher's exact test, $p = 0.018$).

4 | Discussion

We prospectively enrolled 70 TETs who underwent surgery at a single institution over 3 years. Somatic mutations were evaluated by comparing tumor and germline DNA using targeted sequencing specific for TETs, confirming GTF2I as the most frequently mutated gene in TETs.

GTF2I mutation occurred in 41% of tumors, consistent with previous reports (22%–43%) [8–10]. GTF2I mutations were more common in A and AB thymomas and enriched in Stage I–II tumors, supporting their favorable prognosis. At the time of this evaluation, follow-up data were insufficient for survival analysis. These mutations were somatic and occurred at the exact genomic coordinates (chromosome 7 c.74146970T>A, hg19), leading to a leucine-to-histidine substitution at positions 404 and 383 in GTF2I isoforms. GTF2I mutation L404H/L383H is characteristic of TETs, whereas the N440S mutation has been described in endometrial, hepatobiliary, and colon carcinomas. Transcriptome sequencing has shown the expression of GTF2I beta and delta isoforms in TETs [8]. Transgenic mice expressing mutated GTF2I under the FOXN1 promoter develop thymomas, demonstrating that mutated GTF2I is an oncogene [20, 21]. No drugs are currently available to inhibit mutated GTF2I. Fortunately, most GTF2I-mutated TETs are cured with surgical resection.

GTF2I mutation can occur alone or in combination with other mutations. Interestingly, four out of five tumors with an HRAS mutation also presented a GTF2I mutation, although the HRAS variants were noncanonical (A146T, L133H, G13R, K117T, K117N). Conversely, SETD2, DNMT3A, and nonsilent BCOR mutations are more common in tumors with GTF2I wild-type status. ASXL1, TET1, SETD2, DNMT3A, and BCOR are tumor suppressor genes involved in epigenetic regulation and have been associated with TETs; however, their relationship with GTF2I remains unclear. The co-occurrence and mutual exclusivity of GTF2I mutations suggest distinct molecular abnormalities in GTF2I-mutated versus wild-type TETs, potentially explaining the clinical differences between them.

Although thymic carcinomas typically have higher mutation burdens than thymomas [8, 9], we observed no significant differences in the number of mutations. This is likely due to the presence of mutations in genes not included in the 77-gene panel, which was specifically designed based on a cohort enriched in thymomas [10] Previously, we sequenced 67 myasthenia gravis-associated thymomas retrospectively collected between 2015 and 2018 with similar results [10]. No TP53 mutations were observed, which are more frequent in thymic carcinomas, though a CDKN2A frameshift deletion was detected in one out of six thymic carcinomas. CDKN2A/CDKN2B deletions, common in thymic carcinomas, were undetected by our method.

Mutations were absent in 39% of cases, likely due to genes not covered by the panel or the high nonneoplastic cell content in tumor samples, which makes NGS detection challenging. This is common in AB, B1, and B2 histotypes, where tumor cells may represent less than 10% of the sample [1, 22]. In the 19 samples with fresh material for cell dissociation, flow cytometry revealed a very low percentage of epithelial cells, often undetectable or below 1%. While dissociation techniques likely underestimate the proportion of cancer cells, they still indicate a significant dilution of tumor cells. In the TCGA study, over 80% of the BED was covered by more than 20 reads [9]. Petrini I et al. [8] reported an average tumor BED coverage of 68 reads, with 92% covered by at least 20 reads. By focusing on the 77 most mutated genes in TETs, we increased the average coverage to 768 reads, covering 97% of the tumor BED with more than 50 reads. However, somatic mutations were not identified in a substantial portion of TETs. The challenge in obtaining epithelial cells for tumor dissociation, combined with the inefficiency of sorting technologies, hinders cancer cell enrichment. Moreover, TETs exhibit a low tumor mutation burden (0.48 mutations per Megabase) [9]. Current sequencing methods may underestimate the prevalence of INDELs, translocations, and focal copy number changes, which could be relevant to TET biology. Epigenetic dysregulations may be relevant for some TETs, including those without detectable mutations, as preliminary data on miRNA and methylation have been reported [20, 23].

We observed an enrichment of mutations of the COSMIC 25 signature, potentially linked to chemotherapy exposure. Seven patients received chemotherapy before surgery. Mutational signatures varied among reports. Radovich and colleagues described enrichment for signatures 1 (Spontaneous deamination of 5-methylcytosine), 6, and 15 (both Defective DNA mismatch repair), and 14 (Concurrent polymerase epsilon mutation and defective DNA mismatch repair). Our previous review of NGS reports in TETs outlines enrichment for signatures 1, 2 (Activity of APOBEC family of cytidine deaminases), and 5 (unknown etiology). Using a different NGS assay for the same panel of genes, we identified enrichment for signatures 18 (damage by reactive oxygen species), 29 (tobacco chewing), 30 (defective DNA base excision repair due to NTHL1 mutations), 4 (tobacco smoking), 1, and 15. Signature 1, commonly associated with aging, was consistently enriched across analyses, with a median age of diagnosis of 58 years.

In the past decade, significant progress has been made in the molecular characterization and treatment of TETs. The identification of GTF2I as a key driver in most thymomas has provided valuable insights, while thymic carcinomas are frequently associated with mutations in CDKN2A, TP53, and CDKN2B. Our results contribute to a molecular classification of TETs, including thymomas with GTF2I mutation, usually with a favorable prognosis; thymomas without GTF2I mutation, which exhibit more aggressive behavior with local invasiveness and sometimes pleural metastases; and thymic carcinomas, which are frankly aggressive tumors.

Author Contributions

Eleonora Pardini: investigation, formal analysis, validation, and writing – original draft. **Federico Cucchiara:** formal analysis, software. **Serena Barachini:** investigation. **Marina Montali:** investigation. **Gisella Sardo Infirri:** investigation. **Irene Sofia Burzi:** investigation. **Michelangelo Maestri:** methodology, resources. **Melania Guida:** resources. **Roberta Ricciardi:** methodology, resources. **Vanessa Nicolì:** investigation. **Fabio Coppedè:** methodology, project administration, and funding acquisition. **Diana Bacchin:** resources. **Vittorio Aprile:** resources. **Carmelina Cristina Zafira:** resources. **Franca Melfi:** resources. **Marco Lucchi:** methodology, resources. **Iacopo Petrini:** conceptualization, methodology, validation, writing – review and editing, supervision, and funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. A. Marx, J. K. C. Chan, L. Chalabreysse, et al., "The 2021 WHO Classification of Tumors of the Thymus and Mediastinum: What Is New in Thymic Epithelial, Germ Cell, and Mesenchymal Tumors?," *Journal of Thoracic Oncology* 17, no. 2 (2022): 200–213.

2. F. Conforti, M. Marino, V. Vitolo, et al., "Clinical Management of Patients With Thymic Epithelial Tumors: The Recommendations Endorsed by the Italian Association of Medical Oncology (AIOM)," *ESMO Open* 6, no. 4 (2021): 100188.

3. R. J. Kelly, I. Petrini, A. Rajan, Y. Wang, and G. Giaccone, "Thymic Malignancies: From Clinical Management to Targeted Therapies," *Journal of Clinical Oncology* 29, no. 36 (2011): 4820–4827.

4. C. M. S. Proto, G. Galli, M. Imbimbo, et al., "Efficacy and Safety of Ramucirumab Plus Carboplatin and Paclitaxel in Untreated Metastatic Thymic Carcinoma: RELEVENT Phase II Trial (NCT03921671)," *Annals of Oncology* 35, no. 9 (2024): 817–826.

5. G. Giaccone, C. Kim, J. Thompson, et al., "Pembrolizumab in Patients With Thymic Carcinoma: A Single-Arm, Single-Centre, Phase 2 Study," *Lancet Oncology* 19, no. 3 (2018): 347–355.

6. J. Sato, M. Satouchi, S. Itoh, et al., "Lenvatinib in Patients With Advanced or Metastatic Thymic Carcinoma (REMORA): A Multicentre, Phase 2 Trial," *Lancet Oncology* 21, no. 6 (2020): 843–850.

7. C. Proto, S. Manglaviti, G. Lo Russo, et al., "STYLE (NCT03449173): A Phase 2 Trial of Sunitinib in Patients With Type B3 Thymoma or Thymic Carcinoma in Second and Further Lines," *Journal of Thoracic Oncology* 18, no. 8 (2023): 1070–1081.

8. I. Petrini, P. S. Meltzer, I. K. Kim, et al., "A Specific Missense Mutation in GTF2I Occurs at High Frequency in Thymic Epithelial Tumors," *Nature Genetics* 46, no. 8 (2014): 844–849.

9. M. Radovich, C. R. Pickering, I. Felau, et al., "The Integrated Genomic Landscape of Thymic Epithelial Tumors," *Cancer Cell* 33, no. 2 (2018): 244–58.e10.

10. E. Pardini, F. Cucchiara, S. Palumbo, et al., "Somatic Mutations of Thymic Epithelial Tumors With Myasthenia Gravis," *Frontiers in Oncology* 13 (2023): 1224491.

11. K. Kurokawa, T. Shukuya, R. A. Greenstein, et al., "Genomic Characterization of Thymic Epithelial Tumors in a Real-World Dataset," *ESMO Open* 8, no. 5 (2023): 101627.

12. N. Girard, C. Basse, A. Schrock, S. Ramkissoon, K. Killian, and J. S. Ross, "Comprehensive Genomic Profiling of 274 Thymic Epithelial Tumors Unveils Oncogenic Pathways and Predictive Biomarkers," *Oncologist* 27, no. 11 (2022): 919–929.

13. S. Barachini, E. Pardini, I. S. Burzi, G. Sardo Infirri, M. Montali, and I. Petrini, "Molecular and Functional Key Features and Oncogenic Drivers in Thymic Carcinomas," *Cancers* 16, no. 1 (2023): 166.

14. F. C. Detterbeck, A. G. Nicholson, K. Kondo, P. Van Schil, and C. Moran, "The Masaoka-Koga Stage Classification for Thymic Malignancies: Clarification and Definition of Terms," *Journal of Thoracic Oncology* 6, no. 7 (2011): S1710–S1716.

15. A. Jaretzki, R. J. Barohn, R. M. Ernstoff, et al., "Myasthenia Gravis: Recommendations for Clinical Research Standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America," *Neurology* 55, no. 1 (2000): 16–23.

16. Z. Lai, A. Markovets, M. Ahdesmaki, et al., "VarDict: A Novel and Versatile Variant Caller for Next-Generation Sequencing in Cancer Research," *Nucleic Acids Research* 44, no. 11 (2016): e108.

17. J. T. Robinson, H. Thorvaldsdóttir, A. M. Wenger, A. Zehir, and J. P. Mesirov, "Variant Review With the Integrative Genomics Viewer," *Cancer Research* 77, no. 21 (2017): e31–e34.

18. K. Wang, M. Li, and H. Hakonarson, "ANNOVAR: Functional Annotation of Genetic Variants From High-Throughput Sequencing Data," *Nucleic Acids Research* 38, no. 16 (2010): e164.

19. A. Mayakonda, D. C. Lin, Y. Assenov, C. Plass, and H. P. Koeffler, "Maftools: Efficient and Comprehensive Analysis of Somatic Variants in Cancer," *Genome Research* 28, no. 11 (2018): 1747–1756.

20. V. Nicolì and F. Coppedè, "Epigenetics of Thymic Epithelial Tumors," *Cancers* 15, no. 2 (2023): 360.

21. O. B. Giorgetti, A. Nusser, and T. Boehm, "Human Thymoma-Associated Mutation of the GTF2I Transcription Factor Impairs Thymic Epithelial Progenitor Differentiation in Mice," *Communications Biology* 5, no. 1 (2022): 1037.

22. I. Petrini, S. Barachini, V. Carnicelli, et al., "ED-B Fibronectin Expression Is a Marker of Epithelial-Mesenchymal Transition in Translational Oncology," *Oncotarget* 8, no. 3 (2017): 4914–4921.

23. M. Radovich, J. P. Solzak, B. A. Hancock, et al., "A Large microRNA Cluster on Chromosome 19 Is a Transcriptional Hallmark of WHO Type A and AB Thymomas," *British Journal of Cancer* 114, no. 4 (2016): 477–484.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** tca70205-sup-0001-Tables.xlsx.