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Detecting ectopic thymus in thymoma-associated myasthenia gravis through flow cytometry analysis of CD3^{medium}TCRvβ^{medium}CD4^{+}CD8^{+} T cells and its clinical significance

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Background: Traditional histological methods for identifying ectopic thymus (ET) have significant limitations including high risk of false negatives. This study aims to evaluate the effectiveness of flow cytometry in detecting ETs in patients undergoing total thymectomy.

Methods: We analyzed 864 samples from 103 patients using flow cytometry and hematoxylin and eosin (H&E) staining. ETs were identified by the presence of CD3^{medium}TCRvβ^{medium}CD4^{+}CD8^{+} T cells in flow cytometry or Hassall's corpuscles in H&E staining.

Results: In the discovery set, flow cytometry detected ETs in 69.2% of samples, compared to 23.6% by histological methods. The validation set showed a higher incidence of ETs in myasthenia gravis (MG) patients than in non-MG patients (73.5% vs. 58.0%, $P < 0.0001$) and in those with thymic epithelial tumors versus normal thymus (68.1% vs. 58.1%, $P = 0.0088$). MG patients exhibited a higher prevalence of active ETs, characterized by a high proportion of CD4^{+}CD8^{+} T cells, indicating robust thymopoiesis, compared to those without MG ($P = 0.0001$). Specific regions, such as the left cervical root, areas along the right and left phrenic nerves, and the left innominate vein, showed significantly higher activity ($P < 0.05$). Additionally, ETs were more frequently found in the cervical region than in the mediastinum (75.0% vs. 60.8%, $P = 0.0012$), and in patients aged 40 years or younger compared to those older than 40 years (73.0% vs. 60.6%, $P = 0.0027$).

Conclusions: Flow cytometry is a viable alternative for ET detection, providing a novel distribution map that enhances surgical decision-making in MG treatment.

Keywords: distribution, ectopic thymus, flow cytometry, incidence, myasthenia gravis, thymoma

Introduction

Myasthenia gravis (MG) is a well-known autoimmune disease characterized by weakness and fatigability, with ocular and generalized forms being the most common manifestations[1]. Thymic abnormalities are observed in approximately 70–80%

HIGHLIGHTS

- Flow cytometry accurately identify ectopic thymus (ET) in patients with thymoma-associated myasthenia gravis (MG) by detecting CD3^{medium}TCRvβ^{medium}CD4^{+}CD8^{+} T cells.
- A total of 846 samples were detected, ETs were more frequently observed in patients with MG, in younger patients and in the cervical region.
- Patients with MG had a higher prevalence of active ETs, characterized by a high proportion of CD4^{+}CD8^{+} T cells indicating robust thymopoiesis.
- We presented updated distribution patterns of ETs in patients with thymoma, regardless of the presence of MG.

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of MG patients, including thymic hyperplasia, thymomas, and ectopic thymus (ET)[2-4]. Thymectomy, the surgical removal of the thymus, has become an essential treatment for MG as it often leads to symptom improvement or even elimination in most patients[5]. The primary goal of thymectomy is to achieve remission of MG symptoms by removing all thymic tissues. Residual thymic tissues frequently result in incomplete remission of MG symptoms[6,7]. Retrospective studies have shown that more extensive thymus resection leads to higher remission rates and fewer recurrences[8-10]. However, accurate identification of all thymic tissues, particularly ETs, remains challenging, and failure to remove them can lead to incomplete remission.

ET refers to thymic tissue located outside its usual position, often dispersed throughout the mediastinal fat[11,12]. Despite being located in abnormal positions, ETs maintain similar characteristics to normal thymic tissue and contribute to the generation and development of T cells. These tissues, in particular, harbor naïve T cells, especially $CD4^+CD8^+$ T cells with specific marker expression[13]. These widely distributed ETs often play a role in cases where postoperative MG symptoms persist, remission is slow, or recurrence occurs[14,15]. Thus, accurately determining the incidence and distribution of ETs is crucial for effective surgical treatment of MG.

Traditional methods, such as histological examination, have been used in previous studies to examine the distribution pattern of ETs[10,16,17]. However, these methods are time-consuming and can yield false negative results, leading to a lack of consensus on the accurate incidence and complete distribution pattern of ETs. Therefore, there is a pressing need for a new, highly efficient, and sensitive method to diagnose ETs and reassess their distribution.

To address this gap, our initial research focused on a novel method that accurately identifies ETs through flow cytometry analysis of immature $CD3^{medium}TCR\nu\beta^{medium}CD4^+CD8^+$ T cells[18]. The objectives of this study are to validate this novel method's effectiveness, create a more accurate ET distribution map, and investigate factors influencing ET presence, including MG status and age. These findings not only provide valuable insights into the incidence and distribution of ETs but also facilitate better understanding of the extent of resection needed in the surgical treatment of patients with MG.

Materials and methods

Patients and sample acquisition

Suspected ETs were collected from 103 patients who were diagnosed with mediastinal masses and underwent total thymectomy and resection of mediastinal fat tissues at Zhongshan Hospital, Fudan University between January 2017 and September 2022. The collection consisted of a discovery set (n = 182 from 20 patients) and a validation set (n = 664 from 83 patients). All resected tumor tissues were examined by two experienced pathologists to confirm the diagnosis. In the discovery set, samples were obtained from cervical and mediastinal fat tissues, and they were primarily categorized into four groups: above the innominate vein, adjacent to the phrenic nerve, paravascular, and diaphragmatic angle. For further analysis, ten tumoral tissues along with matched peritumoral tissues (located at least 2 cm away from the gross tumor) and five mediastinal lymph nodes were acquired. In the validation set, fat tissues were collected from 12 sampling sites, including the parathyroid, anterior cervical region, right/left cervical root, along the right/left phrenic nerve, right/left heart phrenic angle, left innominate vein, aortopulmonary window, aorto-caval groove, and prepericardium. This study was approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University (B2022-419) and the work has been reported in line with the STROCSS criteria[19].

Tissues and single cells preparation

For improved diagnostic accuracy, the samples were divided equally into two parts. One part was utilized for flow cytometry detection, while the other part underwent immunohistochemistry staining. For flow cytometry analysis, the tissue was first cut into small pieces and thoroughly meshed using a cell strainer. Subsequently, the cells were filtered through a 70-μm cell strainer (Corning, USA) and washed with Roswell Park Memorial Institute (RPMI)-1640 medium (Corning) containing 10% fetal bovine serum (FBS) (Biowest, FRA). After lysing the red blood cells using ACK lysis buffer, the remaining cells were resuspended in RPMI-1640 medium with 10% FBS.

Flow cytometry

For surface marker staining, the cells were washed with staining buffer consisting of PBS containing 1% FBS and 1 mM EDTA. To block non-specific binding, anti-CD16/CD32 antibodies (Fc1) and 10% normal rat serum were added, followed by a 30-minute incubation with surface antibodies on ice. The antibodies utilized for FACS analysis are listed in Supplemental Table 1 (available at: http://links.lww.com/MS9/A700). Dead cells were excluded using the Live/Dead Fixable Aqua Dead Cell staining kit (Thermo Fisher Scientific). The samples were acquired using an LSRFortessa flow cytometer (BD PharMingen) and analyzed with FlowJo software (TreeStar).

Hematoxylin eosin staining and immunofluorescence

Hematoxylin and eosin (H&E) staining and immunofluorescence (IF) were conducted following previously described methods[20]. Briefly, formalin-fixed tissues were dehydrated, rendered transparent, embedded in paraffin, and then sectioned with a thickness of 5 mm. The sections were subsequently deparaffinized, rehydrated, and stained using the H&E staining technique, and representative images were captured under a microscope. For IF analysis, slides were deparaffinized with xylene and rehydrated in a series of ethanol solutions (100%, 95%, 85%, and 70%) for 10 minutes each. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide, followed by antigen retrieval in 0.5 mM EDTA (pH 9.0) buffer through boiling water for 30 minutes. The sections were then cooled naturally to room temperature and blocked with goat serum at routine temperature for one hour. Next, the sections were incubated overnight at 4°C with a mixture of primary antibodies from different species (refer to Supplemental Table 1, available at: http://links.lww.com/MS9/A700). Afterward, corresponding fluorescent secondary antibodies (Alexa Fluor 488 and 594, Yeasen, China) were added and incubated for one hour. Finally, the sections were stained with DAPI containing an anti-fluorescence quenching agent and cover-slipped. Positive staining

was evaluated based on images of 4 representative fields captured under high-power magnification (× 200).

Statistical analysis

Statistical analyses were conducted using SPSS Version 20.0 (For Windows; Chicago, IL, USA). Graphs were generated using GraphPad Prism (Version 9.0.0) or R 4.1.2 statistical software (For Windows; Boston, Massachusetts). For categorical variables, such as the number of different sampling sites, proportions (%) were calculated, and differences between groups were illustrated using histogram and line charts. The proportional compositions of two or more variables were compared using chi-square tests or Fisher's exact tests. All *P* values were two-tailed, and a significance level of $P < 0.05$ was considered statistically significant.

Results

Discovery study of CD20-CD3mediumTCRvβmedium CD4 +CD8+ T cells for detecting ETs

Hematoxylin and eosin (H&E) staining is a commonly used tool for identifying ETs, but its sensitivity is often low, leading to an underestimation of ET distribution. To address this limitation and improve accuracy, we conducted a discovery study using flow cytometry. Our hypothesis was that ETs would contain immature $CD4^+CD8^+$ thymocytes with intermediate levels of CD3 and TCRvβ expression, similar to normal thymic tissue. For the study, we collected 182 tissue samples from 20 patients at the neck and mediastinum regions where ETs were reported (Table 1). Each sample was evenly divided into two parts: one for H&E staining, and the other for flow cytometry analysis (Fig. 1A, Supplemental Figure 1, available at: http://links.lww.com/MS9/A693, and Supplemental Table 2, available at: http://links.lww.com/MS9/A701). In the samples stained with H&E, we used Hassall's corpuscle as an indicator of ET presence, and did find scattered ETs in fat tissue of some samples, with an ET incidence of 23.6%. Intriguingly, in all samples with ETs identified by Hassall's corpuscle, flow cytometry analysis revealed the presence of $CD20^-CD3^{medium}TCRv\beta^{medium}CD4^+CD8^+$ T cells (Fig. 1B, Supplemental Figure 2A–C, available at: http://links.lww.com/MS9/A694). Immunofluorescent (IF) staining further confirmed the presence of $CD4^+CD8^+$ T cells in ET-containing tissues (Supplemental Figure 2D, available at: http://links.lww.com/MS9/A694). Notably, even in cases where thymus structures without Hassall's corpuscles were observed by H&E staining, flow cytometry still detected $CD4^+CD8^+$ T cells (Supplemental Figure 2E,F, available at: http://links.lww.com/MS9/A694). The overall incidence rate of ETs determined by flow cytometry was 69.2%, which was unexpectedly higher than that observed using traditional H&E staining ($P < 0.0001$, chi-square test) (Fig. 1B,C). We also used the results from both methods to establish a composite gold standard: a site was considered positive if it was detected by either method, and negative only if both methods failed to detect it. Sensitivity, specificity, and overall accuracy were then calculated, and the results showed that flow cytometry outperforms traditional immunohistochemical methods in detecting ectopic thymus (Supplemental Table 3, available at: http://links.lww.com/MS9/A702).

Table 1

Clinical pathological characteristics of 103 patients

Characteristics		Total	Percent
Age (year)			
18-40		25	24.3%
>40		78	75.7%
Sex			
Male		51	49.5%
Female		52	50.5%
Tumor size			
>3 cm		43	71.7%
≤3 cm		17	28.3%
Pathological results			
Thymoma A	5		
Thymoma AB	12		
Thymoma B1	10	60	58.3
Thymoma B2	20		
Thymoma B3	7		
Thymic carcinoma	6		
Non-thymic epithelial tumor		43	41.7%
Massaoka stage			
I		20	33.3%
II		20	33.3%
III		14	23.3%
IV		6	10.0%
Myasthenia gravis (MG)			
Yes			
Early-onset MG (EOMG)	13 (43.3%)		
Late-onset MG (LOMG)	17 (56.7%)		
Generalized	20 (66.7%)		
Ocular	10 (33.3%)		
MG medication (>6 m)	11 (36.7%)	30	29.1%
No MG medication (No or <6 m)	19 (63.3%)		
No		73	70.9%
Other autoimmune diseases		4	3.9%
Preoperative chemo- or radiotherapy		8	7.7%
Surgical techniques			
Transsternal/transcervical thymectomy		21	20.4%
(Modified subxiphoid or intercostal) video-assisted thoracoscopic thymectomy		70	68.0%
Robotic-assisted thoracoscopic thymectomy		12	11.6%

To differentiate between ET and other types of tissues, such as thymoma, lymphoid tissue, and fat tissue, we conducted a comparative analysis of their morphological and molecular characteristics using H&E staining, IF staining and flow cytometry analysis. Both ETs and thymomas were found to contain $CD3^{medium}TCRv\beta^{medium}CD4^+CD8^+$ T cells (Fig. 1D and Supplemental Figure 3A,B, available at: http://links.lww.com/MS9/A695), while thymoma tissues demonstrated a distinct demarcation and dense epithelial components in H&E staining, which differed from the appearance of ETs (Supplemental Figure 3B, available at: http://links.lww.com/MS9/A695). In contrast, lymphoid tissues only exhibited $CD3^{high}TCRv\beta^{high}$ mature $CD4^+$ or $CD8^+$ T cells (Fig. 1D and Supplemental Figure 3A,C, available at: http://links.lww.com/MS9/A695), while fat tissues lacked

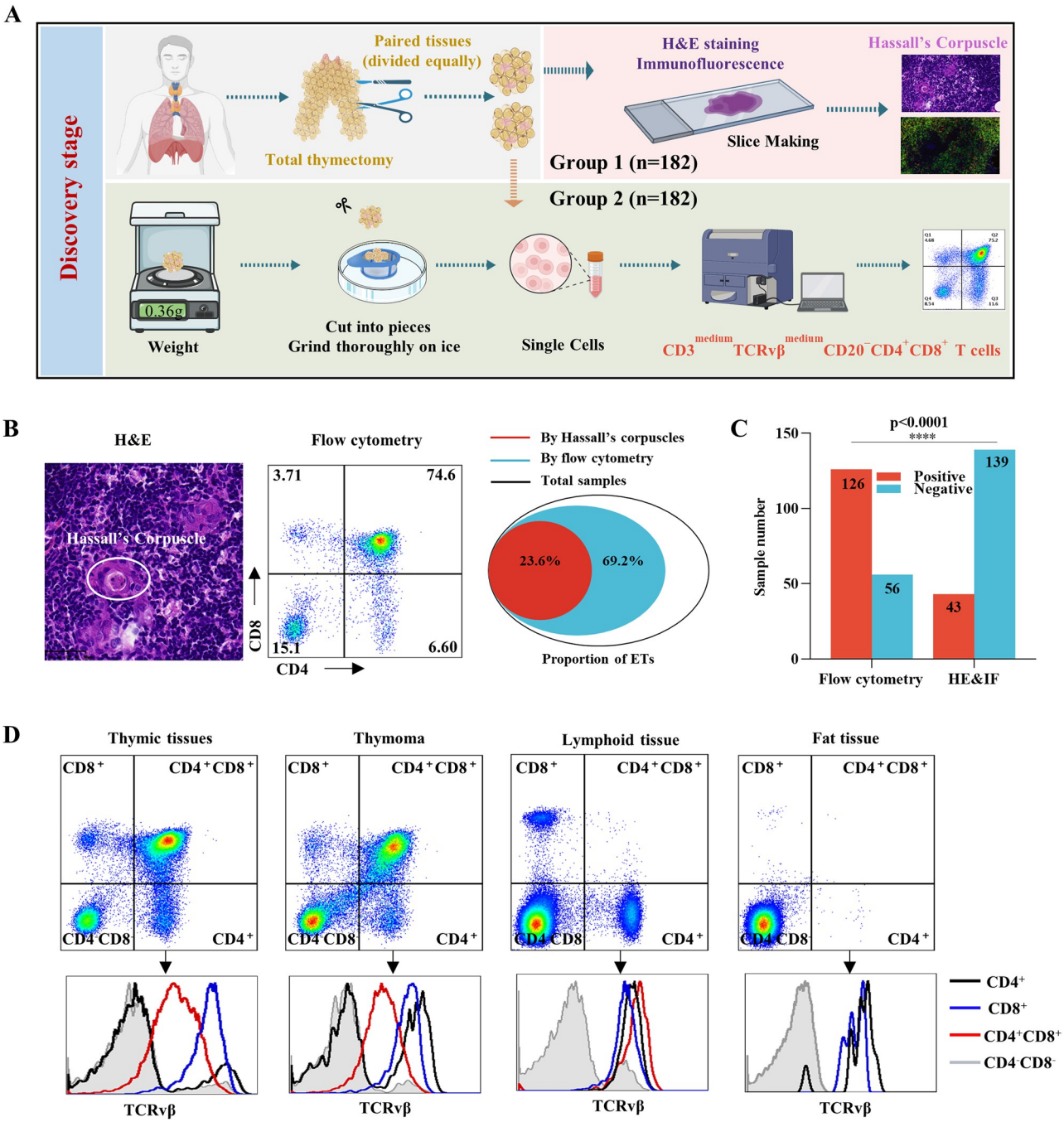

Figure 1. Discovery study of $CD20^{-}CD3^{medium}TCR\nu\beta^{medium}CD4^{+}CD8^{+}$ T cells for the diagnosis of ectopic thymus (ET). (A) Schematic diagram of the design of discovery study. (B) Representative hematoxylin and eosin (H&E) staining of ETs, with an ellipse indicating a Hassall's corpuscle; and flow cytometry analysis of $CD20\text{-}CD3^{medium}TCR\nu\beta^{medium}CD4^{+}CD8^{+}$ T cells in tissues with ETs identified by H&E staining; and the inclusion relationship of ETs detected by Hassall's corpuscle and flow cytometry. (C) Summary and comparison of the number of samples with or without ETs identified by Hassall's corpuscle or flow cytometry by: (D) flow cytometry analysis of CD4 and CD8 expression on cells from indicated tissues (upper panel); and TCRvβ expression on $CD4^{-}CD8^{-}$ cells, $CD4^{+}CD8^{+}$ cells, CD4 single-positive and CD8 single-positive T cells in the indicated tissues (lower panel). The *P* value is indicated by **** ($P < 0.0001$) (chi-square test).

$CD3^{medium}TCR\nu\beta^{medium}CD4^{+}CD8^{+}$ T cells and had a lower presence of mature $CD4^{+}$ or $CD8^{+}$ T cells (Fig. 1D and Supplemental Figure 3D, available at: http://links.lww.com/MS9/A695). In conclusion, our discovery study highlights the reliability and efficiency of flow cytometry-based $CD4^{+}CD8^{+}$ T cell analysis as a highly accurate method for diagnosing ETs.

Validation study of CD20-CD3mediumTCRvβmedium CD4+CD8+ T cells for detecting ETs

To confirm the accuracy of our findings, we employed flow cytometry-based analysis on tissue samples from an independent validation set. The validation set consisted of 664 tissue samples obtained from 83 patients with various thymic diseases,

including 30 cases of myasthenia gravis (Table 1). To ensure consistent and standardized sampling, we developed a customized workflow for tissue and viable cell collection (Fig. 2A). This workflow involved examining twelve different locations covering mediastinal and cervical fat tissues. Schematic diagrams illustrating the front and rear views were utilized to depict these locations (Fig. 2B). The number of sampling locations varied depending on the surgical techniques employed, ranging from three to twelve.

Consistent with our initial findings in the discovery set, the flow cytometry analysis conducted on the validation set (n = 664) revealed a higher prevalence of ET at the twelve sampling sites, ranging from 48.0% to 84.4%, compared to traditional H&E/immunohistochemistry analysis, which showed a range of 3.0% to 72.0% (Fig. 2C, Supplemental Figure 4A, available at: http://links.lww.com/MS9/A696, and Supplemental Table 4, available at: http://links.lww.com/MS9/A703). Amongst the sampling sites, the parathyroid, anterior cervical region, left cervical root and pre-pericardium exhibited a relatively higher incidence of ET, with an occurrence rate exceeding 70%. Conversely, regions along the right phrenic nerve and the right heart phrenic angle displayed the lowest incidence of ET (Fig. 2C).

Interestingly, the overall prevalence of ET was significantly higher in MG patients (73.5%) compared to non-MG patients (58.0%) ($P < 0.0001$, chi-square test), especially along the phrenic nerve and the left heart phrenic angle when compared to patients without MG (Fig. 2D,E). These findings strongly suggest a correlation between MG and ET, as well as specific ET sites associated with MG. Furthermore, we conducted a comparison of the incidence of ET in patients with TET and non-TET. The overall incidence of ET was significantly higher in patients with TET compared with those with non-TET (68.1% vs. 58.1%) ($P = 0.0088$, chi-square test) (Fig. 2F,G, Supplemental Figure 4B,C, available at: http://links.lww.com/MS9/A696). Specifically, we noted a higher incidence of ET at the right cervical root and along the right phrenic nerve in patients with TET (Fig. 2F).

ETs in patients with MG exhibits a higher level of active thymopoiesis

We proceeded to analyze the proportion of $CD4^+CD8^+$ T cells in the ET-positive samples and found a wide range of $CD4^+CD8^+$ T cell proportion. A high proportion of $CD4^+CD8^+$ T cells indicates active thymopoiesis. Based on these proportions, we categorized the ETs into two distinct groups: high activity (≥18.6%), low activity (<18.6%), using a median proportion cutoff of 18.6% (Fig. 3A). Of particular interest, patients with MG had a higher number of ETs exhibiting high activity of thymopoiesis compared to those without MG ($P = 0.0001$, chi-square test) (Fig. 3A, Supplemental Table 5, available at: http://links.lww.com/MS9/A704). To further investigate this phenomenon, we evaluated the proportion of high activity across each sampling location. Remarkably, the left cervical root, regions along the right and left phrenic nerves and the left innominate vein region exhibited a significantly greater proportion of high activity in MG patients compared to those without MG ($P < 0.05$, chi-square test) (Fig. 3B). In terms of the overall proportion of ETs with high activity of thymopoiesis, there was no significantly difference between patients with TET or non-TET. However, a significant difference was observed in the region along the left phrenic nerve (Supplemental Figure 5A,B, available at: http://links.lww.com/MS9/A697). These results suggest that patients with MG not only have a higher incidence of ET but also manifest a greater number of ETs with heightened activity of thymopoiesis.

Clinical relevance of ETs

In addition to considering the presence of MG and the pathological type, we conducted further subgroup analyses to explore potential factors that could influence the incidence of ET. Age emerged as a significant factor, with a higher overall incidence of ET observed in younger patients (age ≤ 40 years) compared to older patients (age > 40 years), with positive rates of 73.0% and 60.6%, respectively ($P = 0.0027$, chi-square test) (Fig. 4A). Interestingly, while age was found to influence the incidence of ET and the incidence of ET was higher in the MG group than in the non-MG group (Fig. 2D), no significant difference was observed between early-onset MG (age <50 years) and late-onset MG (age ≥50 years) ($P = 0.1250$, chi-square test) (Supplemental Figure 6A, available at: http://links.lww.com/MS9/A698). These findings suggest that once patients develop MG, age may not significantly influence the incidence of ET. Furthermore, we compared the incidence of ET in the neck and mediastinum, and discovered that ET was more frequently observed in the neck than in the mediastinum in all patients and patients with MG ($P = 0.0012$ and 0.0192, respectively, chi-square test) (Fig. 4B,C). Additionally, our results indicated that patients with TET had higher incidence of ET in comparison to those with non-TET conditions (Fig. 2F); however, among patients with TET, the incidence of ET showed no significant difference between those with thymomas and thymic carcinomas ($P = 0.3481$, chi-square test) (Supplemental Figure 6B, available at: http://links.lww.com/MS9/A698). Interestingly, among patients with thymoma, the incidence of ET in those with concomitant MG was significantly higher than in those without MG ($P = 0.0172$, chi-square test) (Fig. 4D). In contrast, among patients with MG, the incidence of ET showed no statistical difference between those with thymoma and thymic hyperplasia ($P = 0.6924$, chi-square test) (Supplemental Figure 6C, available at: http://links.lww.com/MS9/A698).

Incidence and distribution of ET in patients with TAMG

Based on our five-year sequential research, we developed distribution maps illustrating the prevalence of ET in distinct patient subgroups. These subgroups include patients with TET (Fig. 5A), thymomatous MG (Fig. 5B), and nonthymomatous MG (Supplemental Figure 7, available at: http://links.lww.com/MS9/A699). In patients with TET but without MG, we observed variable incidence rates of ET at 12 specific locations: 50.0%, 82.4%, 66.7%, 69.2%, 42.1%, 52.4%, 60.9%, 44.0%, 57.9%, 66.7%, 68.8%, and 90.0% (Fig. 5A). Comparatively, patients with thymomatous MG exhibited a higher incidence of ET at almost all 12 locations (Fig. 5B). It is worth noting that although ET was frequently observed in both patient groups, its incidence was notably higher in the MG group. And patients with thymomatous or nonthymomatous

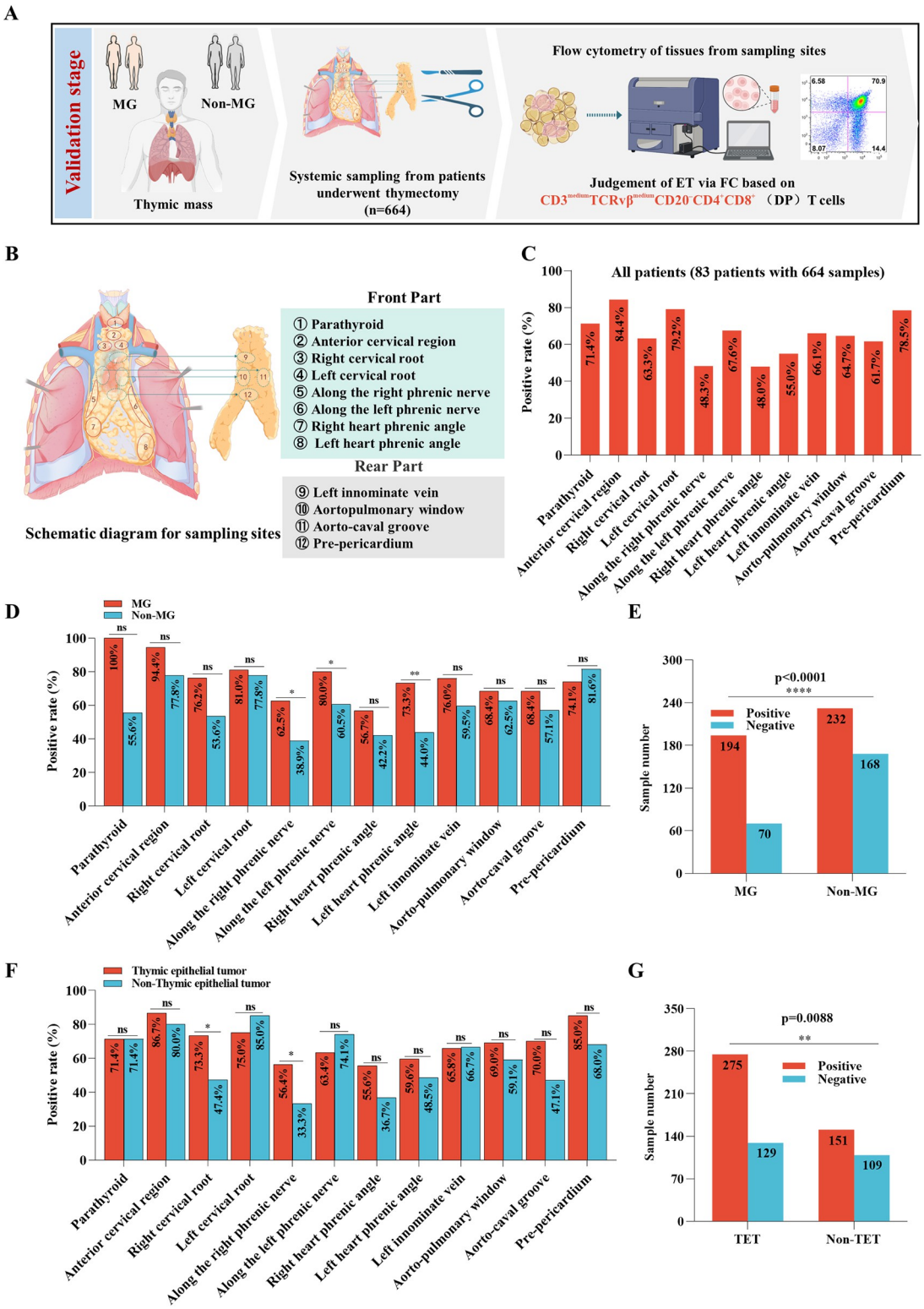

Figure 2. Validation study of CD20-CD3^{medium}TCRvβ^{medium}CD4^{+}CD8^{+} T cells for the ET diagnosis. (A) Schematic diagram illustrating the design of the validation study. (B) Schematic diagram presenting the twelve sampling locations used in the validation study. (C) Summary of the positive rate of ET in samples collected at the twelve indicated locations in the validation study. (D) Summary of the positive rate of ET in samples collected from patients with or without Myasthenia gravis (MG) at the twelve indicated locations. (E) Chi-square test comparing the positive rate of ET between samples from patients with or without MG. (F) Summary of the positive rate of ET in samples collected from patients with or without thymic epithelial tumors (TET) at the twelve indicated locations. (G) Chi-square test comparing the positive rate of ET between samples from patients with or without TET. The *P* values are indicated by n.s. ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$), or **** ($P < 0.0001$) (chi-square test or Fisher's exact test).

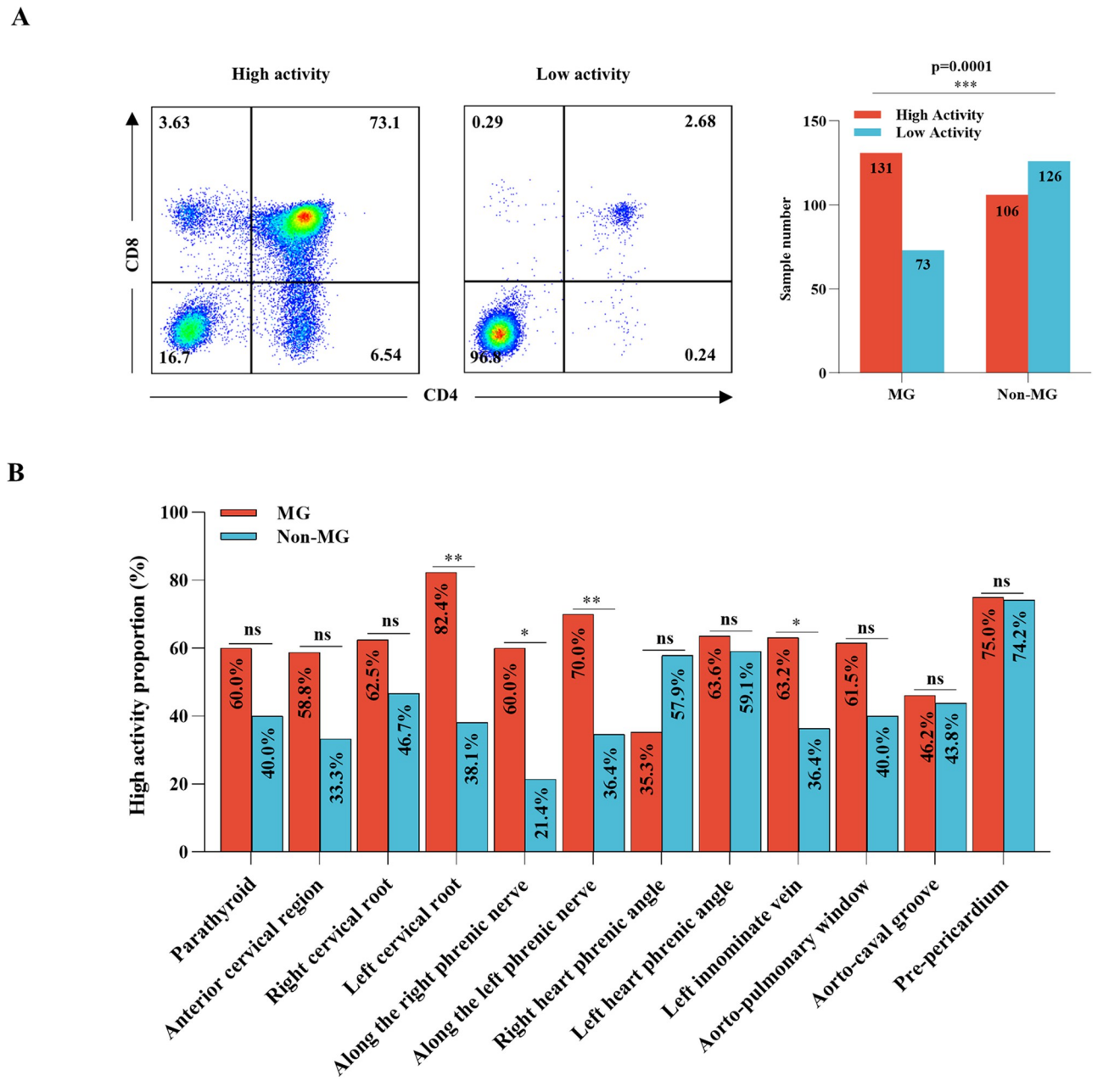

Figure 3. Ectopic thymus in patients with MG exhibits elevated level of active thymopoiesis. (A) Summary of the number of ET samples with high activity of thymopoiesis (proportion of $CD4^+CD8^+$ T cells ≥ 18.6%) and with low activity thymopoiesis (proportion of $CD4^+CD8^+$ T cells < 18.6%) in patients with or without MG. The left panel: representative flow cytometry plots of $CD4^+CD8^+$ T cell in ETs with high or low activity of thymopoiesis, respectively. The right panel: chi-square test comparing the frequency of ETs with high activity of thymopoiesis between patients with or without MG. (B) Summary of the proportion of ET samples with high activity of thymopoiesis at the twelve indicated locations in patients with or without MG. The *P* values are indicated by n.s. ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$) (chi-square test or Fisher's exact test).

MG had similar high incidence of ETs (Fig. 5B, Supplemental Figure 6C, available at: http://links.lww.com/MS9/A698 and Supplemental Figure 7, available at: http://links.lww.com/MS9/A699). Moreover, our analysis identified several specific locations, including the left cervical root, paraphrenic nerve and left innominate vein region, where the activity of thymopoiesis was significantly elevated in patients with MG compared to those without MG (Fig. 5B).

Discussion

The aim of our study was to evaluate the effectiveness of flow cytometry in diagnosing ETs compared to traditional H&E staining. Our findings demonstrate that flow cytometry serves as a reliable method for detecting ETs and offers new insights into their incidence and distribution in thymoma patients with or without MG. The presence of ETs may compromise the efficacy

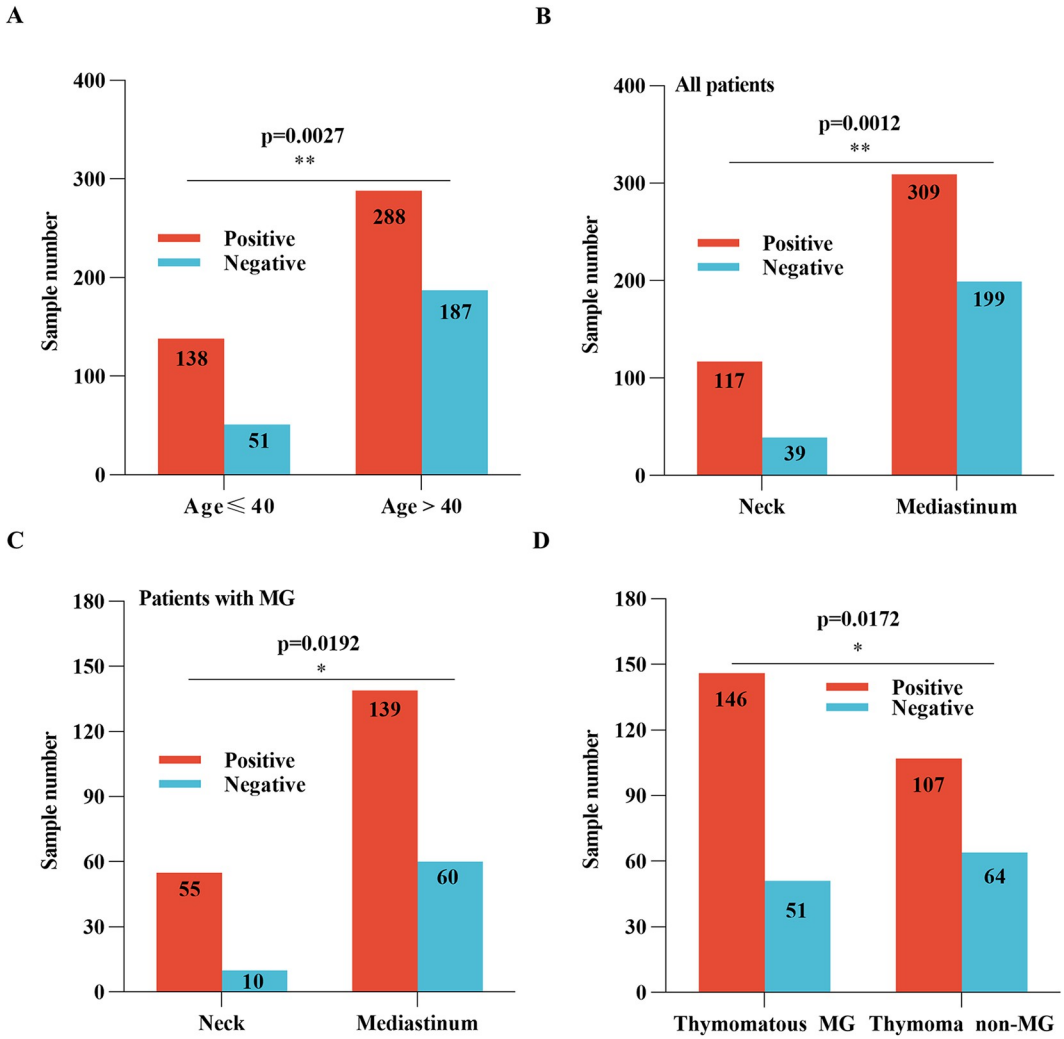

Figure 4. Clinical relevance of ectopic thymus. (A) Summary of the number of ET-positive or ET-negative samples in patients age ≤40 years or >40 years. (B) Summary of the number of ET-positive or ET-negative samples at neck and mediastinal sites. (C) Summary of the number of ET-positive or ET-negative samples at neck and mediastinal sites in MG patients. (D) Summary of the number of ET-positive samples or ET-negative samples at neck and mediastinal sites in thymoma patients with or without MG. The *P* values are indicated by * ($P < 0.05$) or ** ($P < 0.01$) (chi-square test).

of surgical treatment in MG patients[21]. While ETs can occur in various anatomical locations within mediastinal and cervical fat, certain preferred sites allow for maximal thymus resection. Currently, there is no available test to detect the presence of microscopic thymic tissues during or immediately after surgery. Imaging observations of ETs have low sensitivity and specificity, and preoperative CT and MRI scans often fail to detect residual thymus during reoperation[22]. Although immunohistochemistry detection of ETs is accurate, its high false negativity rate is unacceptable[15,23]. Understanding the regulations governing T cell development in the thymic microenvironment provides new valuable insights for identifying hidden ETs in mediastinal fat tissues[24-26]. Our study revealed a notable presence of $CD4^+CD8^+$ T cells in ETs, which can serve as a direct indicator for detecting ETs. To confirm the presence of ETs, additional molecules such as CD3 and TCRvβ were used as auxiliary indicators. If suspected $CD4^+CD8^+$ T cells expressed CD3 and TCRvβ levels between those of double negative (DN) cells and single positive $CD4^+/CD8^+$ T cells, they are categorized as ETs. Conversely, if suspected $CD4^+CD8^+$ T cells expressed CD3 and TCRvβ at levels similar to those of $CD4^+/CD8^+$ T cells, accompanied by a sufficient number of $CD4^+/CD8^+$ T cells, these tissues were likely lymphoid tissues. Suspected $CD4^+CD8^+$ T cells with $CD3^-TCRv\beta^-$ phenotype can be excluded as false positives. Ectopic thymoma was also reported[27,28]; however, its size and morphological features make it distinguishable from ETs. Notably, all ETs identified by H&E staining (presence of Hassall's corpuscles) also exhibited clustered $CD4^+CD8^+$ T cells detected through flow cytometry. Even in tissues where no Hassall's corpuscles were found, $CD4^+CD8^+$ T cells were still detectable using flow cytometry, indicating the significantly higher sensitivity and specificity of flow cytometry in diagnosing ETs.

ETs are recognized as a crucial theoretical basis for surgical treatment of MG. Previous studies have explored the distribution of ET and reported varying incidence rates ranging from 60% to 70% in mediastinal fat tissues, although the anatomical

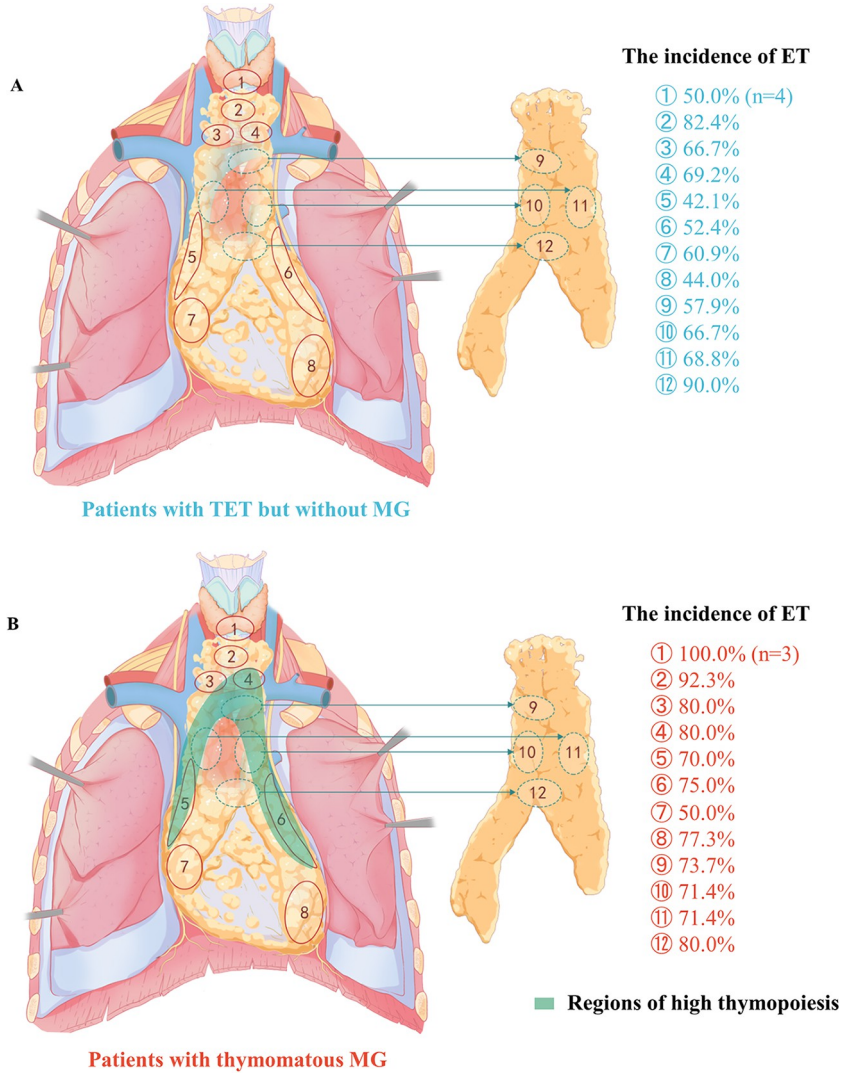

Figure 5. Updated distribution maps of ET in thymoma patients with or without MG based on flow cytometry analysis of immature $CD4^+CD8^+$ T cells. (A) Distribution map of ET in thymoma patients without MG, with the incidence rate of ET at the twelve indicated locations. (B) Distribution map of ET in thymoma patients with MG, with the incidence rate of ET at the twelve indicated locations, and the light green region refers to locations with high thymopoiesis.

regions detected varied between studies[12,21,29-38]. A groundbreaking study conducted by Alfred Jaretzki A III *et al* reported that 32% of mediastinal fat tissues and 22% of cervical fat tissues in patients with nonthymomatous MG contained ET[16]. Other researchers have also provided evidence showing that 45% to 80% of mediastinal fat tissues contain ET[38-40], which is consistent with our findings. By utilizing flow cytometry, we identified a significantly higher incidence of ET compared to previous studies[12,16,21,29,32-34,38]. This discrepancy can be attributed to differences in detection methods. Previous studies mainly relied on staining techniques like H&E or immunohistochemistry, which often fail to detect ectopic thymic foci due to sampling limitations. In contrast, flow cytometry allowed us to comprehensively analyze all cells in the entire tissue, enabling a more accurate and reliable analysis at the single-cell phenotype level. Additionally, variations in patients' symptoms and disease progression might have contributed to the disparity in incidence rates. Our study included samples from patients with abnormal thymus and diverse symptoms, enhancing the generalizability of our findings. Additionally, regional disparities may also play a role, as our study exclusively enrolled Chinese patients.

Further subgroup analyses indicate that the incidence of ET may be influenced by age, suggesting a potential decrease in ET occurrence with thymus degeneration or ET itself. Notably, the incidence of ET in cervical fat tissues is remarkably high, reaching 75.0% (117/156), surpassing most other sampling sites. This proportion is even higher in patients with myasthenia gravis (MG), with a prevalence of 84.6% (55/65). These findings underscore the importance of including cervical fat resection as a crucial aspect of surgical treatment of MG. Previous studies have also recommended maximal resection during reoperation for persistent MG symptoms, emphasizing the significance of formal cervical fat dissection regardless of the surgical approach employed[16,41]. Some studies compared different surgical approaches for ETs resection and emphasized the importance

of thorough resection for the prognosis of MG patients[10,42]. It is worth noting that MG patients exhibit a higher incidence of ET compared to those without MG, while the difference in incidence between early-onset and late-onset MG or between thymomatous and nonthymomatous MG is not statistically significant. Overall, these outcomes imply that MG significantly influences the occurrence of ET, and once MG develops, age and thymus pathology type no longer significantly impact ET incidence. In addition, MG patients tend to have more ETs characterized by heightened thymopoiesis activity compared to those without MG. The presence of $CD4^+CD8^+$ T cells indicates that ET is capable of supporting T cell development. Progenitor T cells migrate to the thymus, where they undergo thymopoiesis, a process through which DN T cells develop into functional $CD4^+CD8^+$ T cells and $CD4^+/CD8^+$ T cells[43,44]. Therefore, a higher proportion of $CD4^+CD8^+$ T cells in ETs reflects more active thymopoiesis. Since functional T cells are crucial for MG development, thymopoiesis is closely linked to the pathogenesis of MG. Among all diagnosed ET samples, some exhibit a prominent $CD4^+CD8^+$ T cell predominance, indicating dense ET population and active thymopoiesis. Particularly, cervical fat tissues, bilateral periphrenic fat tissues, and the left innominate vein region demonstrate a higher proportion of ETs with intense thymopoiesis activity in MG patients, highlighting the necessity of meticulous fat tissue resection within these areas during surgical treatment for MG. For other locations where the incidence and activity of thymopoiesis of ETs are not significantly different, excessive resection may not be necessary.

Thymectomy is a highly effective treatment for curing or alleviating MG, and better outcomes are associated with more extensive thymus resection[17]. However, the extent of thymic resection is often considered complete regardless of the surgical technique used or whether total removal is achieved[45,46]. In clinical practice, the most commonly encountered patients are those with thymomatous MG. The appropriate extent of thymus removal for these patients remains a subject of ongoing debate, with some experts suggesting that removing only the tumor itself may be sufficient. In this study, although the number of patients with nonthymomatous MG is limited, we observed that the incidence of ETs in patients with thymomatous MG was comparable to that in patients with nonthymomatous MG. This suggests that, in addition to thymic tumors, ETs also have a potential impact on the development of MG.

Our study has certain limitations that should be acknowledged. Firstly, due to differences in surgical approaches, we were unable to acquire tissues from all sampling sites of each patient, resulting in insufficient sampling from certain sites, which made reliable statistical analysis challenging and led to potential overestimation or underestimation of positivity rates. Secondly, the sample size of patients with MG in our study was relatively small, limiting our ability to conduct comprehensive subgroup analyses and provide a more precise incidence of ET in patients with nonthymomatous MG. Thirdly, despite including patients from various regions in the country, our study remains a single-center study, which may impact the generalizability of our findings. Therefore, larger-scale multicenter studies with more robust designs are warranted to generate more accurate and widely applicable information. Fourth, flow cytometry may not be available in all centers performing thymectomy, which could limit its widespread application in clinical practice, nonetheless, our findings can provide a reliable reference for clinical application.

Conclusions

In summary, our study provides compelling evidence supporting the use of $CD4^+CD8^+$ T cells as a reliable diagnostic marker for identifying ETs using flow cytometry. We have identified several critical factors associated with ET incidence, including younger age, the cervical site, and the presence of myasthenia gravis (MG). Additionally, we observed a significant correlation between MG status and increased thymopoiesis, particularly in cervical and bilateral periphrenic fat tissues, as well as the left innominate vein region. By employing flow cytometry, we generated an updated distribution map of ETs in patients with thymic diseases, regardless of MG status. These findings enhance our understanding of optimal surgical resection techniques and extent in thymomatous and nonthymomatous MG patients, thereby contributing to the development of improved clinical management strategies.

Ethical approval

This study was approved by the Research Ethics Committees of Zhongshan Hospital, Fudan university (the approval number B2022-419).

Consent

Not applicable.

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Author's contributions

Y.A., J.D., J.J., M.Y.: visualization, investigation, writing – original draft preparation; J.G., S.W. and C.J.: supervision and discussion; Q.L., Y.Z., F.D., Y.Z., J.Z., Y.Z., J.R., Z.Y., Y.S.: software and methodology, formal analysis; A.K., Y.C., H.W., J. D.: conceptualization, writing – review & editing, supervision and funding support.

Conflicts of interest disclosure

The authors declare no competing interests.

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Data availability

All data generated that are relevant to the results presented in this article are included in this article and the supplemental files. Other data that are not relevant for the results presented here are available from the corresponding author Dr. Ding upon reasonable request.

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