Thymoma in Patient with Myasthenia Gravis Has Significantly Fewer Forkhead Box P3 Positive Lymphocytes than that without One

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Summary: Forkhead box P3 (FoxP3) is known as a pivotal and specific transcriptional factor of regulatory T cells, and are implicated in various immune diseases including myasthenia gravis (MG). The aim of this study was to investigate the relationships between FoxP3 expression of lymphocytes in thymoma and clinicopathological characteristics, particularly MG status in thymoma patients.

We reviewed 83 thymoma specimens, including 22 from MG patients, and evaluated the FoxP3 expression of lymphocytes in thymoma using immunohistochemistry (IHC). Statistical association was evaluated using chi-square test and Fisher's exact test.

Thirty-four cases (41.0%) were classified as FoxP3 positive. There were no statistical differences in sex (P=0.289), age (P=0.536), Masaoka stage (P=0.086), WHO histological classification (P=0.097), or Myasthenia Gravis Foundation of America (MGFA) Clinical Classification (P=0.117) between FoxP3 positive and negative cases. In contrast, thymoma in cases with MG showed significantly fewer FoxP3 positive lymphocytes than those in cases without MG (P=0.037). Moreover, cases with anti-acetylcholine receptor antibody titer equal to or greater than the normal limit also showed significantly fewer FoxP3 positive lymphocytes than cases within the normal limit (P<0.001).

Our result indicated the possibility that the decrease of FoxP3 positive lymphocytes in thymoma may lead to the development of MG and to an increase in anti-acetylcholine receptor antibodies. In addition, FoxP3 positive lymphocytes might be a useful biomarker for evaluation of the risk of MG onset, and could open the way to more effective therapeutic strategies in thymoma patients.

Key words regulatory T cell, FoxP3, thymoma, myasthenia gravis

INTRODUCTION

Thymomas are derived from thymic epithelia and they have a variety of histological features. In 1999, the World Health Organization (WHO) published the histological classification system for thymic tumors [1], and a detailed reclassification was done in 2004 [2].

Thymomas are classified mainly into five subtypes, A, AB, B1, B2, and B3. Type A thymomas are thought to derive from the medulla, type B from the cortex, and type AB are a mixed type. Type B thymomas are further subdivided into B1, B2, and B3 depending on the percentage of lymphocytes and extent of epithelial cell atypia [3,4].

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Abbreviations: Ach-R, acetylcholine receptor; FFPE, formalin-fixed paraffin-embedded; FoxP3, forkhead box P3; IHC, immunohistochemistry; MG, myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; Tregs, regulatory T cells; Tris-EDTA, Tris-ethylent diamine tetra acetic acid; WHO, World Health Organization.

The most common paraneoplastic phenomenon associated with thymoma is myasthenia gravis (MG), which affects 25–45% of thymoma patients [4-6]. Type B thymomas in particular are frequently associated with MG [7]. Myasthenia gravis is an autoimmune disease caused by CD4$^+$ T-cell-dependent development of anti-acetylcholine receptor (Ach-R) antibodies that cause muscle weakness by impairing neuromuscular transmission [8-10]. Ströbel et al. [11] reported that intra-tumorous naive CD4$^+$ T cells were present in thymomas associated with MG, and concluded that paraneoplastic MG is closely associated with the capacity of thymomas to produce and export naive CD4$^+$ T cells.

Regulatory T cells (Tregs), which are one of the subtypes of CD4$^+$ T cells, have been shown to play a critical role in the prevention of organ-specific autoimmunity and allograft rejection. Tregs exert an autologous inhibitory effect on the activation of antigen-specific T cell subsets and can control self-reactive T cell responses [12-14]. Recent studies have shown that the maturation of CD4$^+$CD25$^+$ Tregs was impaired in MG-associated thymoma [15]. In addition, forkhead box P3 (FoxP3) is considered to be a specific transcriptional factor required for the differentiation and development of Tregs, and is widely used as a specific marker of this subset [16,17].

We investigated FoxP3 positive lymphocytes in thymoma tissue samples using immunohistochemistry (IHC), and surveyed correlations between its expression and clinicopathological characteristics including MG, anti-Ach-R antibody titer, and the severity of MG symptoms.

MATERIALS AND METHODS

Patients

We reviewed formalin-fixed paraffin-embedded (FFPE) tissue samples from 83 cases of thymoma which underwent thymectomy at Kurume University Hospital between 1990 and 2007. Pathological diagnoses were performed by two pathologists (NT and OK) in all cases, based on the 2004 WHO histological classification. Clinical data were reviewed by reference to the patient's clinical charts.

IHC and definition of Foxp3 expression

A block containing the broadest available surface of tumor tissue was cut into 4 μm sections. The detailed protocol of IHC of FoxP3 was as follows: Deparaffinized sections were pretreated with the aim of antigen retrieval in a microwave oven at 95°C for 40 min in tris-ethylenediaminetetraacetic acid buffer (Tris-EDTA buffer). 3% H$_2$O$_2$ solution was used to blockade the endogenous peroxidase activity. Samples were incubated for two hours in the primary anti-FoxP3 antibody (1:40, mouse monoclonal, PCH101, eBioscience, San Diego, USA) at room temperature. The secondary antibody of Envision System polymer (Dako, Tokyo, Japan) was used for 30 min at room temperature. Staining was visualized by incubation with diaminobenzine (Dako) [18]. In this technique, we used tissue samples of pericardium as a negative control and confirmed the experimental validity.

Two investigators (NT and OK) evaluated the FoxP3 expression without knowing the clinical details of each case. The number of FoxP3 positive lymphocytes was counted by an optical microscope at a magnification of ×400, and the number of positive cells was averaged over five individual viewing fields. Cases with more than 10 FoxP3 positive lymphocytes per field were defined as positive cases, and cases with 10 or less cells per field were defined as negative cases.

Statistical analysis

JMP software (SAS Institute, Cary, NC, USA) was used for all statistical analyses in this study. The chi-square test was performed for comparison of clinico-pathological classifications and FoxP3 expression, although Fisher's exact test was adapted when necessary. A P value <0.05 was considered to indicate a significant difference.

RESULTS

Clinicopathological characteristics

Patient's characteristics of the 83 thymoma cases are shown in Table 1. There were 35 males and 48 females with a median age of 56 years. Thymomas were histologically classified into type A in three cases (3.6%), type AB in 25 cases (30.1%), type B1 in 21 cases (25.3%), type B2 in 25 cases (30.1%), and type B3 in nine cases (10.8%) in accordance with the 2004 WHO histological classification. Twenty-two cases (26.5%) were complicated with MG, with no case (0%) in type A, four cases (16.0%) in type AB, eight cases (38.1%) in type B1, seven cases (28.0%) in type B2, and three cases (33.3%) in type B3. Furthermore, the elevation of anti-Ach-R antibody titer was observed in 28 cases (33.7%), with no case (0%) in type A, five cases (50.0%) in type AB, seven cases (53.9%) in type B1, 12 cases (75.0%) in type B2, and four cases (57.1%) in type B3. All MG cases showed the eleva-

TABLE 1.
Patient Characteristics

Variables	Number of patients (n = 83)	%
Sex		
Male	35	42.2%
Female	48	57.8%
Age (years)		
Median [range]	56 [27-78]	
< 56	40	48.2%
≥ 56	43	51.8%
Masaoka stage		
I	41	49.4%
II	27	32.5%
III	10	12.0%
IVa	0	0.0%
IVb	5	6.0%
WHO classification		
Type A	3	3.6%
Type AB	25	30.1%
Type B1	21	25.3%
Type B2	25	30.1%
Type B3	9	10.8%
MG		
Present	22	26.5%
Absent	61	73.5%
Anti-Ach-R antibody titer (nmol/L)		
< 0.2	20	24.1%
≥ 0.2	28	33.7%
Unknown	35	42.2%
MGFA Clinical Classification (MG cases, n = 22)		
I	3	13.6%
IIa	14	63.6%
IIb	4	18.2%
IIIa	0	0.0%
IIIb	0	0.0%
IVa	0	0.0%
IVb	0	0.0%
V	1	4.5%

Abbreviations: WHO, World Health Organization; MG, myasthenia gravis;
Anti-Ach-R, anti-acetylcholine receptor; MGFA, Myasthenia Gravis Foundation of America.

tion of anti-Ach-R antibody titer, as did six cases (9.8%) without MG. MG cases were categorized as class I in three cases (13.6%), class IIa in 14 cases (63.6%), IIb in four cases (18.2%), and V in one case (4.5%), according to the Myasthenia Gravis Foundation of America (MGFA) Clinical Classification, which is an internationally accepted severity scoring system of MG.

Immunohistochemical FoxP3 expression of lymphocytes in thymoma

In this study, the FoxP3 was expressed in 34 cases

FoxP3 IHC

	Low magnification	High magnification

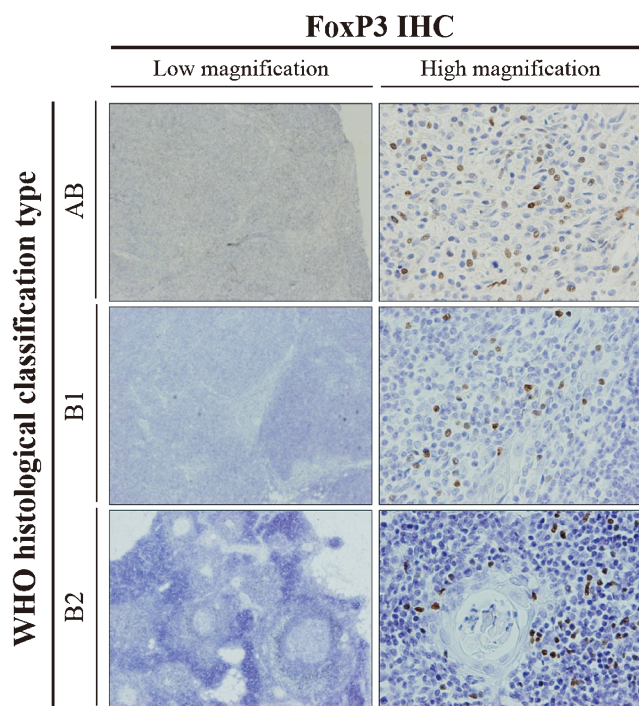WHO histological classification type

AB

B1

B2

Fig. 1. Representative microphotographs of immunohistochemistry in FoxP3 positive expression cases. Type AB case (upper row), type B1 case (middle row), and type B2 case (lower row) are presented. Immunohistochemistry of FoxP3 revealed positive expression in over 10 lymphocytes per field at magnification ×400. (left column: magnification ×40, right column: magnification ×400)

(41.0%), all of which showed infiltrating lymphocytes with diffusely stained nuclei but no cytoplasmic staining (Fig. 1). FoxP3 expression was not observed in any of the epithelial tumor cells. Furthermore, FoxP3 expression of lymphocytes revealed almost uniform staining proportion in the whole specimen. Only five cases with MG (22.7%) were found to be FoxP3 positive, in contrast with 29 cases (47.5%) in cases without MG. Additionally, in terms of anti-Ach-R antibody titer, 13 cases (65.0%) were determined as FoxP3 positive at less than the normal limit (<0.2 nmol/L), while only five cases (17.8%) were determined as positive at equal to or more than the normal limit (≥0.2 nmol/L).

Statistical association between FoxP3 expression of lymphocytes and clinicopathological features in thymoma

The statistical association between FoxP3 expression of lymphocytes in thymoma and clinicopathological features is shown in Table 2. Cases with MG showed significantly fewer FoxP3 positive lymphocytes than those without MG in thymoma (P=0.037).

Moreover, cases with anti-Ach-R antibody titer equal to or greater than the normal limit also showed significantly fewer FoxP3 positive lymphocytes compared with cases having less than the normal limit (P<0.001). In contrast, there were no significant differences in sex (P=0.289), age (P=0.536), Masaoka stage (P=0.086), WHO histological classification (P=0.097), and MGFA Clinical Classification (P=0.117).

DISCUSSION

We found that 41.0% of the thymoma tissue samples evaluated in the present study contained FoxP3 positive lymphocytes in IHC. We detected significantly fewer FoxP3 positive lymphocytes in thymoma of MG patients than in those without MG. In addition, we also demonstrated that there are significantly fewer FoxP3 positive lymphocytes in thymoma patients with elevated anti-Ach-R antibody titer than in those without anti-Ach-R antibody elevation.

Sakaguchi et al. [19] were the first to propose Tregs as CD4+CD25+ cells in 1995, which emphasize the suppressive role in immune response in the periphery, following elucidation of its key transcriptional factor FoxP3 [16]. Gene transfer of *FoxP3* converts naive CD4+CD25- T cells into a regulatory phenotype in both mice and humans and is required for Tregs function [20,21]. Accumulated evidence shows that Tregs actively suppress the activation and expansion of self-reactive T cells by cell-to-cell attachment, thereby preventing autoimmune disease [22,23]. It is also reported that the depletion of CD4+CD25+ T cells from normal animals leads to spontaneous development of various autoimmune diseases such as autoimmune gastritis, thyroiditis, and type 1 diabetes, as well as inflammatory bowel disease [24].

We found that thymomas in MG patients contained significantly fewer FoxP3 positive lymphocytes than those in patients without MG. This result suggests that FoxP3 positive lymphocytes have the ability to contribute to suppression of autoimmune reaction, as can be seen in the other autoimmune diseases described above. Furthermore, Fattorossi et al. [25] suggested that a deficiency of CD4+CD25+Tregs favors the development of MG and that their normalization is an important clinical benefit of immunosuppressive therapy. Their considerations and our results propose that the evaluation of FoxP3 expression in lymphocytes in thymoma might be a predictive biomarker for risk evaluation of MG onset, and could lead to a new therapeutic strategy in MG patients.

This study also showed that thymomas with ele-

TABLE 2.
Statistical analysis between clinicopathological features and FoxP3 expression

Variables	FoxP3 positive (n = 34)	FoxP3 negative (n = 49)	P
Sex			
Male	12	23	0.289
Female	22	26	
Age (years)			
< 56	15	25	0.536
≥ 56	19	24	
Masaoka stage			
I / II	31	37	0.086
III / IV	3	12	
WHO histological classification			
Type A/AB	15	13	0.097
Type B1/B2/B3	19	36	
MG			
Present	5	17	0.037*
Absent	29	32	
Anti-Ach-R antibody titer (nmol/L) **			
< 0.2	13	7	< 0.001*
≥ 0.2	5	23	
MGFA Clinical Classification (MG cases, n = 22)			
I	2	1	0.117
IIa/IIb/V	3	16	

Abbreviations: WHO, World Health Organization; MG, myasthnia gravis; Anti-Ach-R,
anti-acetylcholine receptor; MGFA, Myasthenia Gravis Foundation of America.
*P < 0.05, ** excluded unknown cases

vated anti-Ach-R antibody titer had significantly fewer FoxP3 positive lymphocytes than those without elevated anti-Ach-R antibody. This fact suggests that the down-regulation of FoxP3 of lymphocytes may lead to the production of anti-Ach-R antibody, which then results in the pathogenesis of MG. Wang et al. [26] also reported that a decreased number of circulating CD4⁺CD25⁺ Tregs in MG patients was associated with the positive-MG sera of anti-Ach-R antibody titer, although the relationship between FoxP3⁺ lymphocytes in the periphery and the anti-Ach-R antibody titer remains uncertain. Their report and our current results indicate that a decrease of some population of Tregs is present in both the microenvironment of thymoma and in the systemic circulation in MG patients. These results also have the potential to lead to better clinical follow-up and therapeutic strategies in MG patients.

On the other hand, FoxP3 expression in lymphocytes in thymoma of MG patients was not associated with MG severity, according to the MGFA Clinical Classification. However, this result may be due to the small sample size in our study, because class IIa/IIb/V cases obviously showed a tendency to have fewer FoxP3 positive cases (3/19, 15.8%) as compared with class I cases (2/3, 66.6%) (Table 2). Moreover, the frequency of FoxP3 positive lymphocytes in thymoma was associated with anti-Ach-R titer in our study, as we have described. The association between clinical severity and anti-Ach-R antibody titer remains controversial, although some reports suggested the usefulness of repeated anti-Ach-R antibody measurements in evaluation of clinical development of MG [27]. No studies have examined the association of FoxP3 expression of lymphocytes in thymoma with MG and clinical MG severity, therefore further studies including a large number of MG cases may resolve this question, and could lead to clinical initiatives for the treatment of MG.

Our study has some limitations. First, we surveyed only FoxP3 expression as a specific marker of Tregs.

In general, Tregs have been widely defined as CD4$^+$ CD25$^+$ lymphocytes in past studies, although recent studies have clarified that FoxP3 is a master regulator of Treg cell function [28]. In addition, it has been reported that the frequency of FoxP3 positive lymphocytes compared with CD4$^+$CD25$^+$ T cells in peripheral blood was lower in patients with MG than in those without it [29], which suggests that FoxP3 plays a special role in Tregs. Further study of FoxP3 expression in lymphocytes in thymoma from MG patients including CD4 and CD25 might provide helpful information for elucidating the biological interaction of Tregs in the thymoma microenvironment. Second, we investigated FoxP3 expression only with IHC. More investigations including *FoxP3* gene analysis would develop the results of our study. Third, our study had a small sample size, especially in MG cases. Future studies should include a larger sample size of MG cases enabling the further elucidation of relationships between FoxP3 expression and MG status, including symptoms, anti-Ach-R receptor antibody titer, and MGFA Clinical Classification.

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