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ORIGINAL ARTICLE

Pathological Findings in Myasthenia Gravis Patients with Thymic Hyperplasia and Thymoma

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Abstract Thymectomy is routinely carried out in patients with myasthenia gravis (MG) and thymomas. However, there is still a dispute as to whether MG patients with thymic hyperplasia should undergo thymectomy. We aimed to investigate the pathological findings in the thymus in patients with co-existing MG and thymic hyperplasia or thymomas treated with thymectomy, as well as effects of immunosuppression. Thirty-three patients with MG were selected and grouped accordingly: patients with no thymic abnormalities, patients with thymic hyperplasia, and patients with thymomas. All patients were treated with methylprednisolone alongside immunosuppression. A separate cohort of 24 MG patients with thymic hyperplasia or thymomas and treated with thymectomy were selected. As controls, 5 patients with thymomas or thymic carcinoma without MG were selected. Expression of CD5, extracellular regulated protein kinases1/2 mitogen activated protein kinase (ERK1/2MAPKs) and CD95 ligand (FasL) in the thymus was examined. Methylprednisolone and immunosuppressive therapy are highly effective in MG patients with normal thymus tissue and MG patients with thymic hyperplasia compared to MG patients with thymomas alone. CD5 expression was highest in MG patients with thymic hyperplasia, correlating with expression of ERK1/2MAPKs. FasL expression was similar across all groups. Thymomas may be distinguished from thymic hyperplasia by expression of CD5 and ERK1/2MAPKs. Thymectomy is the preferred treatment for MG patients with thymomas but may not be necessary in MG patients with thymic hyperplasia who are treated with immunosuppressive therapy.

Keywords Myasthenia gravis · Thymic hyperplasia · Thymoma · Thymectomy

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Introduction

Myasthenia gravis (MG) is a relatively rare neurological autoimmune disorder characterized by autoantibodies against the acetylcholine receptor (AChR) or other proteins of the neuromuscular junction [1, 2]. Approximately 80% of patients with MG show thymic abnormalities. The association of MG with thymic abnormalities, such as thymic hyperplasia and thymoma, is found in 60–75% of patients, and is a peculiar feature of the disease [3]. This association, together with the finding that thymectomy reduces the amount of circulating anti-AChR antibodies (Abs) and the rate of clinical relapse, suggests that the thymus plays an important role in the onset and/or maintenance of MG [4, 5].

Alongside surgical treatments, immunosuppressive therapy has been shown to be associated with increased survival rates and improved quality of life in MG patients. Alongside pharmacological therapies, thymectomy has been shown to demonstrate improvements in symptoms, or even remission of MG in a high proportion of patients [6]. It has been demonstrated that the AchRα subunit is only expressed in MG patients with thymomas and is not expressed in patients with thymomas alone. This suggests that the autoantibody response seen in MG patients may be influenced by the presence of a thymoma [7].

Thymectomy is the primary indicated treatment for MG patients with thymomas. However, whether thymectomy in patients with MG and thymic hyperplasia is effective is still a point of contention [8–10]. One recent evidence-based review failed to show conclusive benefits of thymectomy in non-thymoma MG patients, and only recommended thymectomy as an option to increase the probability of remission or symptom improvement [11]. The activation of a variety of immune responses in pathologically different MG phenotypes may account for this finding.

CD5 is an antigen expressed on mature T cells and the B-1 alpha subgroup of lymphocytes. It plays an important role in the selection and development of T cells in the thymus [12, 13]. Increased CD5 expression, within a certain threshold, can prevent the onset of autoimmune disease [14]. However, abnormal CD5 expression deregulates negative selection of T cells occurring in the thymus, leading to autoimmune disease due to the production of autoreactive T cells [15, 16].

T cell activation is correlated with the extracellular regulated kinase 1/2 and mitogen activated protein kinase (ERK1/2MAPK) signaling pathway, and abnormalities in MAPK signaling are associated with thymic hyperplasia in MG patients. Deregulation of these processes is a key step in the pathological remodeling of the myasthenic thymus [17]. Furthermore, CD5 has been shown to down-regulate FasL expression, increasing the propensity of autoreactive T cells [18, 19]. FasL induced cell death (ICD) signaling impairs activation-induced proliferation in B and T cells by diminishing phosphorylation of phosoplipase C-γ (PLCγ), protein kinase C (PKC) and ERK1/2 [20, 21]. Interaction between Fas and FasL provides the key signal for maintaining apoptosis of immune cells, a major mechanism of peripheral tolerance induction and immune homeostasis [22]. Indeed, humans deficient in Fas and FasL exhibit wide-spread systemic autoimmunity [23].

This study aimed to examine the correlation between the expression of CD5, ERK1/2 MAPK, and FasL in thymic tissues from MG patients with varying pathological subtypes, and to investigate the possible pathological mechanisms among MG patients treated with and without thymectomy.

Methods

Experimental Design and Subjects

Fifty-seven patients with MG and 5 non-MG patients were retrospectively analyzed from January 1st, 2002 to December 31st, 2009, at Xijing Hospital and First Affiliated Hospital of Medical College of Xi'an Jiao Tong University, China. Written informed consent was obtained from all patients or their legal surrogates. The study was approved by the Ethics Committee in each respective hospital. The diagnosis of MG was confirmed by MG diagnostic criteria with three absolute requirements including: fluctuating weakness and fatigue of voluntary muscle, positive prostigmine or Tensilon test, and no symptoms implicating any other neurological disease. Supportive criteria of a MG diagnosis included decreased complex action potential during stimulation of the corresponding nerve. Exclusion criteria for patients included: increased complex action potential when the corresponding nerve was stimulated by high frequency electric stimulation, Lambert-Eaton syndrome, mitochondrial encephalomyopathy, Fisher syndrome, and thyrotoxic periodic paralysis.

Thirty-three patients were selected based on the following criteria: MG patients with normal thymus (n = 12), thymic hyperplasia (n = 16) and thymoma (n = 5) treated with methylprednisolone and azathiopurine, ciclosporin A, or tacrolimus alone. Twenty-four patients with MG, presenting with thymic hyperplasia (n = 9) and thymomas (n = 15), and 5 non-MG patients with thymomas or thymic carcinoma treated with thymectomy were also selected.

Medication

Adults were initially treated with methylprednisolone (1 g) daily. Dosage was halved every 3 days, with treatment ending on day 27. Pediatric patients were treated with methylprednisolone (500 mg), along with an immunosupressant such as azathiopurine (1.5-3 mg/Kg/d), cyclosporinA (14–17.5 mg/Kg/d), or tacrolimus (0.15–0.3 mg/kg/d).

Recording Method

Physicians specialized in Myasthenia Gravis Foundation of America (MGFA) scoring recorded patient scores every morning before administering pyridostigmine. MGFA scores were recorded prior to treatment and at 3 d, 1 week and 6 weeks post-treatment [24].

MGFA Clinical Classification

The clinical grade of disease severity was evaluated according to MGFA clinical classification. Specifically, the clinical grades were: class 1, ocular muscle weakness only; class 2,

mild generalized weakness; class 3, moderate generalized weakness; class 4, severe generalized weakness; and class 5, intubation required [24].

Expression of CD5, FasL, ERK1/2MAPK in the Thymus of Patients MG

Formaldehyde-fixed and paraffin embedded human thymus or thymoma/thymic carcinoma tissue was retrieved from the Department of Pathology. Sections, (2 μm thickness) were prepared according to routine protocols. Briefly, sections were heated for 1 h at 55 °C, de-paraffinated in xylene three times and rehydrated in ethanol as follows: 2 × 100% *v*/v, 1 × 95% *v*/v, 1 × 90% *v*/v, and 1 × 70% *v*/v. Antigen retrieval was performed by boiling slides at 600 watt (3 × 5 min) in a microwave oven using citrate buffer at pH 6.0 (Target Retrieval Solution, Dako, USA). Endogenous peroxidase activity was blocked by applying 0.3% hydrogen peroxide. Sections were incubated with 2% bovine serum albumin or blocking serum of the same species as the biotinylated secondary antibody. Sections were stained using rabbit monoclonal antibody against CD5 (Abcam, Cambridge, UK), mouse monoclonal antibody against ERK1/2MAPK (Abcam, Cambridge, UK), and rabbit polyclonal antibody against FasL (Abcam, Cambridge, UK), and incubated for 48 h at 4 °C. Following incubation, slides were incubated with donkey anti-goat, or goat anti-rabbit IgG (1:500, Sigma), at room temperature (20–25 °C) for 2 h, and ABC complex (1:500, Sigma) at room temperature (20–25 °C) for 2 h. The nickel-intensified diaminobenzidine (DAB) reaction was used to detect peroxidase activity. Sections were observed under a light microscope (BX-60, Olympus, Tokyo, Japan). Digital images were captured using specific software (LSM; Zeiss, Oberkochen, Germany) and were printed using a true-color printer (Pictro 3000; Fuji Photo Film Co, Tokyo, Japan).

Evaluation of Immunoreactivity

Immunoreactivity analysis was performed by 3 observers blind to the diagnosis of both the patient and antibodies used for staining. A well-established published scoring system was adopted [24]. A total of 100 randomly selected fields from each slide were viewed under the microscope (×400). The number of positive cells in each field was counted with Image-Pro Plus, and the average number of positive cells in 100 fields was calculated.

Statistical Analysis

The repeated measure variance analysis was performed to evaluate differences between the groups of 33 patients with MG treated with methylprednisolone and immunosuppressive therapy with respect to expression of CD5, ERK1/2MAPK, and FasL. Linear correlation was performed to evaluate correlation of expression of CD5 and ERK1/2MAPK. Two-tailed *P*-values <0.05 were considered statistically significant.

Results

Thirty-three patients with MG who were treated with methylprednisolone and immunosuppressive therapy without thymectomy were included in this study. The cases were comprised of 14 males and 19 females with a mean age of 30.9 years (range 8–69 years) at disease onset. According to Osserman criteria [25], this cohort was comprised of eight type I cases, four type IIa cases, eleven type IIb cases, four type III cases, five type IV cases, and one type V case. Two patients exhibited Graves disease, one patient exhibited hyperthyroidism, one patient exhibited rheumatoid arthritis, and one patient exhibited Sjogren syndrome. Twenty-five patients were tested with low and high frequency repetitive nerve stimulation: 18 cases were positive for low frequency repetitive nerve stimulation. The mean time to disease onset was 2.8 years (range 23 days - 7 years). Thirty-three patients were examined with contrast-enhanced computed tomography (CT) scanning. Upon reviewing the CT scans, 12 MG patients displayed no thymic abnormalities and 16 MG patients displayed thymic hyperplasia. Five MG patients showed round or oval masses in the thymus, with a uniform density, defined border, and no invasion of the mediastinum, and were considered to have thymomas.

Physicians specialized in MGFA scoring recorded baseline scores before daily morning administration of pyridostigmine in MG patients treated with methylprednisolone and immunosuppressive therapy. MGFA scores pre-treatment and post-treatment at six weeks were recorded **(**Fig. 1**)**. MGFA scores in patients with MG with no thymic abnormalities were compared to patients who had MG and thymomas using repeated measure variance analysis. These data suggest that curative effects increase with treatment time. The disease curative effects were improved within a week in MG patients with non-thymic abnormalities and thymic hyperplasia. These effects were significantly improved within four weeks. The curative effect was superior to MG patients with thymomas **(**Table 1**)**. These data suggest that methylprednisolone and immunosuppressive therapy are highly effective in MG patients with normal thymus and MG patients with thymic hyperplasia compared to MG patients with thymomas alone**.**

We next sought to investigate the molecular basis for the immunosuppressive therapy findings observed between experimental groups. We chose another cohort of 24 patients, which included: MG patients with thymic hyperplasia ($n = 9$), MG patients with thymomas not treated with methylprednisolone or immunosuppressive therapy ($n = 15$), and patients without MG with thymomas or thymic carcinoma as

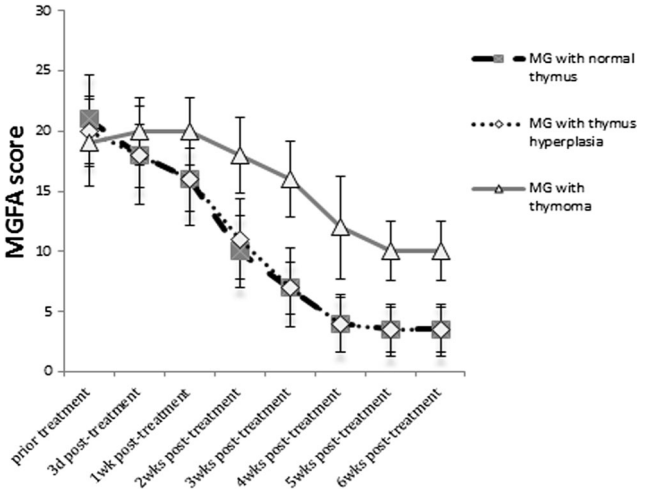

Fig. 1 Short-term treatment with methylprednisolone and immunosuppressive therapy reduced MGFA scores in MG patients with normal thymus , thymic hyperplasia, and thymomas. MGFA scores were recorded every morning before pyridostigmine administration prior to treatment and 3d, and 1 to 6 weeks post-treatment in MG with normal thymus (n = 12), MGFA scores were 21 ± 3.69, 18 ± 2.59, 16 ± 2.59, 10 ± 2.98, 7 ± 2.19, 4 ± 2.26, 3.5 ± 2.20, and 3.5 ± 2.20, respectively. In MG patients with thymic hyperplasia (n = 16), MGFA scores were 20 ± 2.90, 18 ± 4.07, 16 ± 3.80, 11 ± 3.34, 7 ± 3.26, 4 ± 2.36, 3.5 ± 1.86, and 3.5 ± 1.86, respectively. In MG patients with thymoma (n = 5), MGFA scores were 19 ± 3.63, 20 ± 2.83, 20 ± 2.83, 18 ± 3.16, 16 ± 3.16,12 ± 4.24,10 ± 2.45, and 10 ± 2.45, respectively

a control group (n = 5). Thymectomy was performed in all 29 patients in this group. Table 2 shows the clinical data of 29 patients treated with thymectomy. This patient group included 5 males and 4 females with a mean age of disease onset of 25 years (range 15–68 years) in MG with thymus hyperplasia. According to Osserman type, this patient set was comprised of three type I cases, two type IIa cases, and four type IIb cases. One patient exhibited Graves disease and one patient exhibited hyperthyroidism. Nine patients were tested with repetitive frequency and neostigmine tests, all of which were positive. The mean disease onset course was 47.7 months (range 1–240 months). The results of CT in 9 patients demonstrated punctiform aggrandizement rather than mass like enhancement, confirming diagnosis of MG with thymic hyperplasia. Five patients developed myasthenia crisis poster-operatively within three days and were treated with methylprednisolone and intravenous human immunoglobulin. MG patients with thymomas included 8 males and 7 females, with a mean age of disease onset of 46 years (range 19–64 years). According to Osserman criteria, this patient set was comprised of two type I cases, four type IIa cases, four type IIb cases, four type III cases, and one type IV case. One patient exhibited Graves disease. Nine patients were tested with repetitive frequency and neostigmine test, all of which were positive. The mean disease onset course was 7.1 months (range 0.2–40 months). Analysis of the CT scans in 15 patients showed round or oval masses in the thymus, with a uniform density, defined border, and no invasion of the mediastinum and were considered to have thymomas. Two patients developed myasthenia crisis post-operatively within three days and were treated with methylprednisolone and intravenous human immunoglobulin. Expression of CD5, ERK1/2MAPK, and FasL in thymic tissues was investigated following thymectomy **(**Fig. 2**)**. As shown in Fig. 2, CD5 was mainly localized in the cell membrane and FasL was mainly localized in the cytoplasm. ERK1/2MAPK was mainly located in the nucleus. The number of CD5 positive cells was 140.1 ± 33.3 in MG patients with thymic hyperplasia, and CD5 expression was mainly located in the corpuscles of the thymus. The numbers of CD5 positive cells were 89.2 ± 37.1 and 73.2 ± 53.4 in MG patients with thymomas and non-MG with thymomas or thymic carcinoma, respectively. The number of ERK1/2MAPK positive cells was 139.1 ± 55.4 in MG patients with thymus hyperplasia, and ERK1/2MAPK expression was concentrated in the corpuscles of the thymus. The numbers of ERK1/2MAPK positive cells were 37.7 ± 42.7 and 29 ± 24.2 in MG patients with thymomas and non-MG patients with thymomas or thymic carcinoma, respectively. CD5 expression correlated with ERK1/2MAPK expression in thymic tissues (linear correlation analysis r = 0.586, P < 0.05).

Table 1 Repeated measure variance analysis of MGFA scores with short-term treatment in MG patients

Three group sum of squares	df	Mean square	F	*P* value	
30,639.153	1	30,639.153	596.796	0.000	
891.804	2	445.902	8.685	0.001	
1,540.182	30	51.339			
Source of variation	Variation	Sum of squares of mean diviation	Mean square	F value	*P* value
Group	1	891.804	445.902	8.685	0.001
Time point	7	6453.518	3619.859	375.84	<0.001
Time*group	14	286.221	27.587	11.246	<0.001

Repeated measure variance analysis results suggest that the variation in MGFA scores in the three groups differed with time of treatment (P<0.001). Curative effects improved with increase in treatment time. The curative effect of normal thymus and thymic hyperplasia groups with MG was improved at a week, and was significantly improved at 4 weeks. The curative effect is superior than that of thymoma with MG (P = 0.001)

Table 2 Clinical profiles of 29 patients undergoing thymectomy

	Thymus hyperplasia-MG (n = 9)	Thymoma-MG (n = 15)	Non-MG (n = 5)
Sex	Male 5	Male 8	Male 3
Onset age of onset (yr),rang	25 (15 ~ 68)	46 (19 ~ 64)	56 (33 ~ 65)
Disease duration (month)	47.7 (1 ~ 240)	7.1 (0.2 ~ 40)	7.8 (1 ~ 26)
Osserman type			
I ocular type	3	2	
IIa mild type	2	4	
IIb moderate systemic type	4	4	
III acute severe type		4	
IV delayed severe type		1	
Repetitive frequency test	+	+	
Neostigmine test	+	+	
Thymus enhancement CT	Punctiform enhancement	Mass enhancement	Mass enhancement
Lung-function test:			
0 normal	3	7	2
1mildrestrictive/obstructive	3	3	2
2midrange obstructive	3	2	1
3severe obstructive		3	
Postoperative myasthenia crisis	5	2	

The numbers of FasL positive cells were 60 ± 89.6, 32.1 ± 66.7, and 41.6 ± 88.6 in MG patients with thymic hyperplasia, MG patients with thymomas, and non-MG patients with thymomas or thymic carcinoma, respectively. These data suggest molecular differences between MG subtypes, with MG patients with thymomas exhibiting similar expression levels of the aforementioned markers compared to non-MG patients with thymomas or thymic carcinoma. These differences may result in the variance of drug treatment efficacy observed between patient cohorts.

Discussion

Myasthenia gravis is a relatively rare autoimmune disease with a prevalence of 1 in 12,500 worldwide [26]. MG prognosis is influenced by multiple factors such as the presence of a thymoma or generalized thymic hyperplasia. There exists an abnormal immunological state in patients with and without pathological changes of the thymus gland [1]. The number of circulating lymphocytes and architecture of the thymus plays an important role in the pathogenesis of MG and determines disease severity [2, 3]. It has been demonstrated that the AchRα subunit gene has three DNA restriction fragments. It is only expressed in MG patients with a thymoma and not expressed in patients presenting with a thymoma without MG. It has been suggested that the AchR autoantibody response may stem from the thymoma [7]. Thymectomy is a necessary treatment for MG patients with a thymoma. However, thymectomy may not be necessary for MG patients with thymic hyperplasia. It has been previously reported that large doses of methylprednisone result in atrophy in hyperplastic thymus tissue and can successfully induce disease remission [27, 28].

MG patients with thymic hyperplasia usually have a complete and stable disease remission [29, 30]. It is an important clinical question as to whether thymectomy should be performed for all MG patients with an abnormal thymus. The difference in the molecular mechanisms between MG patients with thymic hyperplasia and thymomas may be accounted for by the expression of varying signaling molecules. In our study, CD5 was highly expressed in MG patients with thymic hyperplasia. Thus, our data suggest that CD5 plays the most important role in disease pathogenesis. Previous studies have shown that CD5 plays a key role in the selection and development of T cells in the thymus, in particular, by inhibiting T Cell Receptor (TCR) signaling pathways [12, 13]. Indeed, an increase in CD5 expression, within a certain threshold, has been shown to prevent the onset of autoimmune disease [14]. Accumulating evidence indicates that T lymphocyte activation and selection is sensitive to variations in the expression level of CD5. Abnormal expression of CD5 results in deregulation of negative selection, leading to release of autoreactive T cells into the periphery [15]. Work by Ragheb et al., [14, 16, 31] divided patients into two groups: MG patients and healthy controls. This study demonstrated that there was no significant difference between the number of CD5 T cells in patients with MG and healthy controls. However, the number of CD5 B cells in patients with MG was slightly increased compared to healthy controls. Thus, these findings

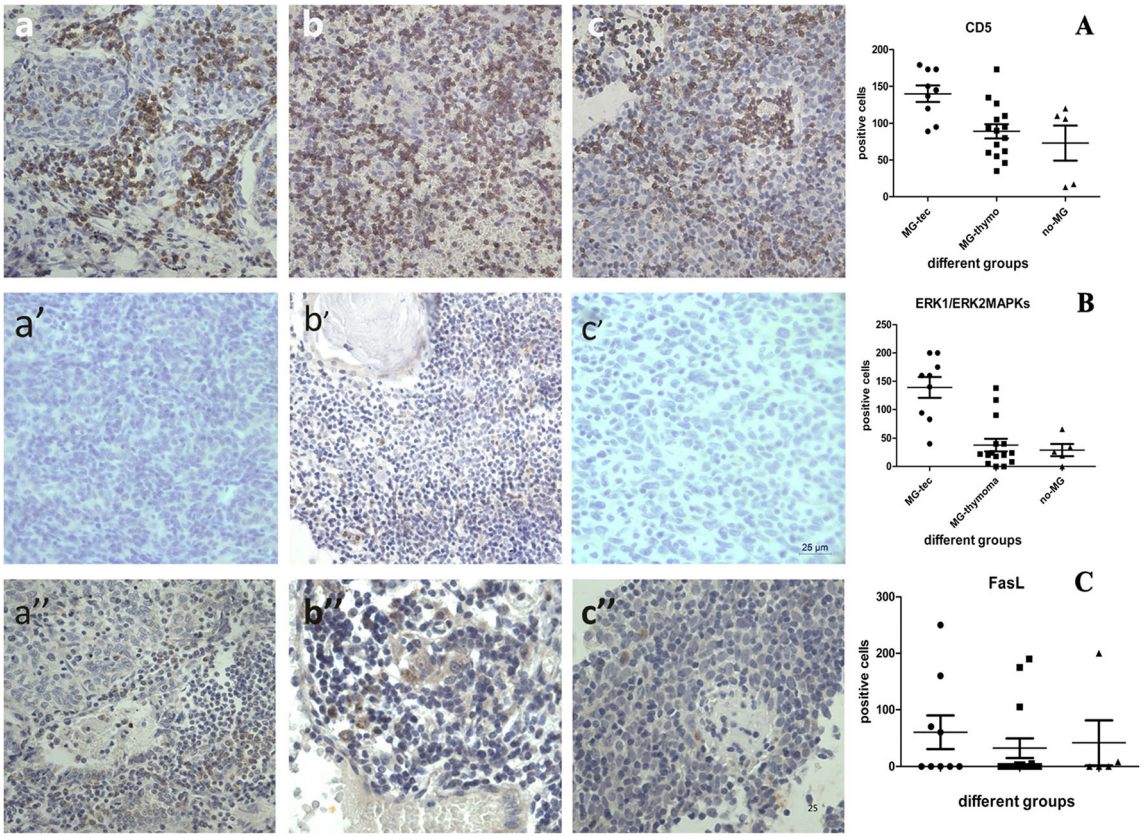

Fig. 2 Expression of CD5, ERK1/2MAPK and FasL in non-MG thymoma/thymic carcinoma patients, MG patients with thymic hyperplasia and thymomas. CD5, ERK1/2MAPK, and FasL expression was detected in thymic tissue from non-MG patients with thymomas or thymic carcinoma (a,a',a"), MG patients with thymus hyperplasia (b,b',b"), and MG patients with thymomas (c,c',c"), respectively. Scale bar: 25 μm. **A.** The number of CD5 positive cells was 140.1 ± 33.3 in MG patients with thymic hyperplasia, and expression was concentrated in the thymus corpuscles. The numbers of positive cells were 89.2 ± 37.1 and 73.2 ± 53.4 in MG patients with thymomas and non-MG patients with thymomas or thymic carcinoma respectively ($F = 6.4, P < 0.05$). **B.** The number of ERK1/2MAPK positive cells was 139.1 ± 55.4 in MG-thymic hyperplasia. The numbers of positive cells were 37.7 ± 42.7 and 29 ± 24.2 in MG-thymoma and non-MG-thymoma/thymic carcinoma, respectively ($F = 16.570$, $P < 0.05$). **C.** The number of FasL positive cells was 60 ± 89.6, 32.1 ± 66.7, and 41.6 ± 88.6 in MG-thymic hyperplasia, MG-thymoma and non-MG-thymoma/thymic carcinoma, respectively ($F = 0.361$, $P > 0.05$). CD5 expression correlated with ERK1/2MAPK expression in the thymus ($r = 0.586$, $P < 0.05$)

did not implicate CD5 in the pathogenesis of MG. In our study, patients were divided into three groups: MG patients with thymic hyperplasia, MG with thymomas, and non-MG-thymoma or thymic carcinoma patients. Our results suggest that CD5 plays an important role in the pathogenesis of thymic hyperplasia in MG patients.

Colombara et al. [17] demonstrated that p38 and ERK1/2 proteins were overexpressed in MG patients with thymic hyperplasia. Indeed, the expression of MAPK subtypes was different in varying pathological subtypes of MG. The activation of T cells correlated with the ERK1/2MAPK signaling pathway. Our study showed that expression of ERK1/2MAPK was significantly higher in MG patients with thymic hyperplasia compared to MG patients with thymomas. Furthermore, our results suggest differences in T cell activation between MG patients with thymic hyperplasia and MG patients with thymomas. Indeed, the transduction of the suppression signal of CD5 may correlate with ERK1/2MAPK signaling. Previous findings have suggested that both MAPK transcriptional and posttranscriptional abnormalities in MG patients with thymic hyperplasia play a role in pathological remodeling of the thymus [23]. We speculate that the initiating factors of autoimmunity in MG patients with thymic hyperplasia and MG patients with thymomas may vary, due to the expression of different cell surface molecules and activation of alternating signaling pathways.

Furthermore, CD5 down-regulates FasL expression, which induces proliferation in B and T cells, leading to a propensity of an autoimmune disease state [18, 19]. Our results also demonstrated that FasL expression in MG patients with thymomas and thymic hyperplasia was consistently low. Maintance of FasL-mediated apoptosis and activation-induced cell death (AICD) of autoreactive T cells is a major mechanism of peripheral tolerance induction and immune homeostasis [22]. The importance of Fas signaling in immune homeostasis is illustrated by the significant accumulation of peripheral T cells

and the development of systemic autoimmunity in both mice and humans deficient in Fas and FasL [23, 32]. In our study, abnormalities in Fas-mediated apoptosis were shown in all MG patients with thymomas, thymic hyperplasia, and non-MG patients with thymic tumor/cancer through differing signaling pathways.

Conclusions

Our study highlighted the molecular mechanisms that may be involved in MG. Furthermore, our study provides a strategy for treating MG patients with different etiologies. We suggest that using CT-guided thymic biopsy prior to thymectomy, and analyzing samples for CD5, ERK1/2MAPKs and FasL expression may help stratify patients [33, 34]. Future studies should examine larger cohorts and further prove the molecular mechanisms involved in MG pathogenesis.

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Author Contributions Dr. Ping Chen, Ying-Peng Wang, Dan-lei Mou: study concept, analysis, literature review, and initial draft of paper; Dr. Zheng-Yi Li, Qiu-Min Qu, Ting Wang, Li-Hua Chen: acquisition of data for literature search, clinical scoring and analysis; Dr.Hong-Yan Wang, Yuan Deng, Xiao-Feng Li: tissue processing, immunohistochemistry experiments and analysis; Dr. Xian-hao Xu, Gang Zhao: critical revision of manuscript for intellectual content and clarity.

Compliance with Ethical Standards

Conflict of Interests None to declare.

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