Journal of Neuroimmunology 221 (2010) 101–106

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Ectopic and high CXCL13 chemokine expression in myasthenia gravis with thymic lymphoid hyperplasia

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ARTICLE INFO

Article history:
Received 29 June 2009
Received in revised form 14 January 2010
Accepted 15 February 2010

Keywords:
Myasthenia gravis
CXCL13
Chemokine
Gene expression microarray

ABSTRACT

Myasthenia gravis (MG) is an antibody and complement mediated autoimmune disease. Serum CXC chemokine ligand 13 (CXCL13) was found to be elevated in MG patients and high CXCL13 level was associated with severe clinical stages, especially in females with thymic lymphoid hyperplasia. Both protein and mRNA of CXCL13 and CXC chemokine receptor 5 (CXCR5) in the thymic tissues were significantly higher in MG patients with lymphoid hyperplasia than those with thymoma. Our data indicated that serum CXCL13 can be used as an index of disease severity and ectopic thymic expression of CXCL13 might be associated with aberrant cell trafficking to the thymus of MG.

1. Introduction

Myasthenia gravis (MG) is characterized clinically by progressive weakness of skeletal muscles on prolonged exercise, with rapid restoration of power after physical activity is discontinued. It has been estimated that the prevalence of MG is 50 to 125 per million population in the United States (Kurtzke and Kurland, 1992). The age of disease onset is much younger in women (20–40 years of age) than in men (50–60 years of age) (Drachman, 1994). MG is generally considered to be an autoimmune disease. Autoantibodies to nicotinic acetylcholine receptor (AChR) can be detected in 85% of MG. Although the trigger of autoimmunity in MG is unknown, it is well documented that the thymus plays an important role in the pathogenesis of MG (Hohlfeld and Wekerle, 1994; Onodera, 2005). Thymic pathological changes commonly observed in MG patients include lymphoid hyperplasia and thymoma. Thymectomy has been reported to offer clinical improvement for some patients (Papatestas et al., 1971; Buckingham et al., 1976). However, not all patients showed beneficial effects after the surgery (Gronseth and Barohn, 2000; Vincent et al., 2001). Thus, the variable histological changes in the thymus and their correlations with clinical manifestations suggest that MG might arise through different pathogenic pathways (Onodera, 2005).

Cytokines and chemokines are proteins responsible for the communication between different cells in the immune system. Several cytokines have been shown to be associated with MG, for example, interleukin (IL)-10, IL-6, interferon (IFN)-γ, IL-12 and interferon-γ inducible protein (IP)-10 (Poussin et al., 2000; Tüzün et al., 2005; Yoshikawa et al., 2006; Feferman et al., 2005). Recently, several studies have reported the role of chemokines, CXC chemokine ligand 13 (CXCL13) and CC chemokine ligand 21 (CCL21), in MG pathogenesis (Meraouna et al., 2006; Le Panse et al., 2006; Saito et al., 2005). Presumably, aberrant expression of chemokine genes could

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doi:10.1016/j.jneuroim.2010.02.013

trigger abnormal migration of immune cells and, consequently, augment pathogenic immune reactions in local lymphoid tissues (Roy et al., 2002; Carlsen et al., 2004).

Under physiologic conditions, CXCL13 is produced by cells in the lymph nodes, spleen, omentum and by peritoneal microphages (Ansel et al., 2002). CXCL13 is required for the development of secondary lymphoid organs, such as the spleen, Peyer's patches and lymph nodes. Mice deficient in CXCL13 and its receptor CXCR5 also failed to form lymphoid follicles (Förster et al., 1996; Gunn et al., 1998; Ansel et al., 2000). In addition to directing B cell homing to lymphoid follicles in secondary lymphoid organs, CXCL13 is also required for B1 cells homing to the omentum and the peritoneal body cavity (Ansel et al., 2002). B1 cells are a specialized subset of B cells, which can be distinguished from the majority of circulating conventional B cells (B2 cells) by their tissue distribution, cell surface markers, and unique function (Hayakawa and Hardy, 2000). They have been reported to be involved in autoantibody production and development of autoimmune diseases (Hayakawa et al., 1983; Hardy and Hayakawa, 2001). Increased numbers of B1 cells have been observed in Sjögren's syndrome and rheumatoid arthritis (Dauphinée et al., 1988; Plater-Zyberk et al., 1985; Barone et al., 2005). Experiments with the murine SLE model have also demonstrated that ectopic and over expression of the B1 cell attractant, CXCL13, can be of pathological significance (Sato et al., 2004; Ito et al., 2004). The higher expression of CXC chemokine receptor 5 (CXCR5) in B1 cells than in B2 cells can explain B1 cell homing to tissues expressing the B cell chemoattractant, CXCL13 (Ishikawa et al., 2001).

To understand the pathogenesis of MG, we have investigated the role of chemokines and chemokine receptors in mediating cell trafficking and local immune reactions in the thymus. We set out to analyze serum cytokines using protein arrays and examined CXCL13 expression in the thymus. In this paper we report the high and ectopic CXCL13 expression in MG pathogenesis.

2. Materials and methods

2.1. Sample collection

One hundred and ninety-four patients were recruited from the Shin Kong Memorial Hospital, Taipei, Taiwan. Thymic tissue specimens were collected from patients who underwent thymectomy. Informed consent was obtained from each participant under protocols approved by the Institutional Review Board of the Shin Kong Memorial Hospital, Taipei, Taiwan. Non-MG thymic tissues were obtained from autopsy samples in the Department of Pathology, Buddhist Tzu-Chi General Hospital, Hualien, Taiwan. Two hundred and thirty-three health subjects (including eighty-six males and one hundred and forty-seven females) were collected as controls with gender and age matched to the patient group. The demographic data of cases and controls are shown in Table 1.

2.2. Cytokine antibody array

One hundred and twenty different cytokines were screened simultaneously using the Human Cytokine Antibody Array C Series 1000 Kits (RayBiotech, Inc. USA). Sera from patients and control subjects were diluted 1:100 and incubated with array membranes according to the manufacturer's instructions. After washing, membranes were incubated with cocktail of biotin-conjugated antibodies. Antibodies with horseradish peroxidase (HRP)-conjugated streptavidin were used as the secondary antibodies. After washing away the unbound antibodies and incubating with detection buffers, chemiluminescence signals were detected by Kodak Biomax MR Film (Kodak, New York, USA). The films were scanned by Microtek ArtixScan 2500f (Microtek International Inc., HsinChu City, Taiwan). The signal intensity of each cytokine was read by GenePix Pro 6.0 software (MDS Analytical Technologies Inc., USA) and normalization was done by positive controls in the antibody arrays.

Table 1
Demographic data of cases and controls.

Group		*N*	Mean age years (range)	Mean CXCL13 pg/ml (range)
Case	Male	65	48.32 (22–95)	81.14 (15–1225)
	Female	129	45.78 (11–91)	120.86 (5–725)
	Total	194	46.63 (11–95)	107.55 (5–1225)
Control	Male	86	50.74 (26–89)	94.19 (20–345)
	Female	147	47.62 (23–86)	111.77 (16–575)
	Total	233	48.77 (23–89)	105.28 (16–575)

2.3. Enzyme-linked immunosorbent assay (ELISA) for CXCL13

The concentrations of CXCL13 in sera were determined by sandwich ELISA. Samples with 1:5 dilutions were added to 96-well flat-bottomed microtiter plates and incubated for 90 min at room temperature according to the manufacturer's instructions (RayBiotech, Inc. USA; detection limit, 1.5 pg/ml). Optic absorbance was measured at wavelength 450 nm using SpectraMax Plus (Molecular Devices, Sunnyvale, CA, USA). A standard curve was generated with a log–log graph using the SOFTMax PRO software (Molecular Devices, Sunnyvale, CA, USA).

2.4. Immunohistochemical staining

Tissues obtained from thymectomy were fixed with 10% formalin, embedded in paraffin and cut into 5-μm sections for immunohistochemical staining. Samples were de-waxed, re-hydrated through stepwise handling with graded ethanol treatments, and washed in double-distilled water. Antigen retrieval was carried out by the Microwave Vacuum Histoprocessor RHS-1 (Milestone Inc., Shelton, CT, USA) before staining. Endogenous peroxidase activity was quenched by 3% hydrogen–peroxide–methanol solution. Sections were incubated with murine anti-human CXCL13 monoclonal antibodies (R&D Systems, Minneapolis, MN, USA), diluted at 1:20 in 0.15 M phosphate buffered saline (PBS) or rabbit polyclonal anti-human CXCR5 antibodies (Abcam Ltd., Cambridge, UK), diluted at 1:100 in PBS at 4 °C overnight. Streptavidin–alkaline phosphatase (ALP) IgG conjugate was added following incubation with biotinylated antibodies (DAKO Cytomation LSAB2 System-HRP; DAKO, Copenhagen, Denmark). Color development was carried out by the DAB Substrate Kit (DAKO Cytomation Liquid DAB + substrate chromogen system; DAKO, Copenhagen, Denmark).

2.5. Real-time PCR analysis

Total RNA was isolated from thymus tissues of MG patients using TRI REAGENT (Sigma-Aldrich, USA) according to the manufacturer's instructions. Control thymus RNA was purchased from Ambion (Ambion Inc., USA) and BioChain (BioChain Institute, Inc., USA). Sample N_Thymus_A was from a 71 year-old male donor obtained from Ambion (Cat#7964). Sample N_Thymus_B1 and N_Thymus_B2 were from BioChain (Cat#R1234-50-CDP) and they were male donors with an age of 23 and 21, respectively. Transcript concentration of CXCL13, CXCR5, and ACTB (beta-actin) were measured through quantitative PCR of complementary DNA. Reverse-transcribed reaction of RNA was carried out by Transcriptor Reverse Transcriptase (Roche Applied Science, Germany). TaqMan Gene Expression assays (CXCL13: Hs00757930_m1, CXCR5: Hs00173527_m1 and ACTB: Hs99999903_m1; Applied Biosystems, Foster City, CA) were used for real-time fluorescence detection using the ABI 7900HT system (Applied Biosystems, Foster City, CA). The gene expression level of CXCL13 and CXCR5 in each sample was normalized with that of ACTB.

2.6. Statistical analysis

Continuous trait (serum CXCL13 and age) were taken the nature log (Ln) to fit the normal distribution for further parametric analysis. Statistical analysis was performed by the correlation analysis or General Lineal Model (GLM) test using SPSS15 for window software (SPSS Inc. USA). A value of $p<0.05$ was considered significant.

3. Results

As an initial screen, cytokine antibody array was applied to identify candidate markers for MG. From a total of 120 cytokines we selected 22 targets for further analysis by ELISA. These 22 targets were selected by their association of clinical features (gender, onset class, clinical stage and thymus pathology) in the pilot study. The remaining 98 were not pursued further. Among the candidates, we have analyzed 10 targets (IL-8, BNDF, CXCL13, CNTF, Eotaxin-2, IL1A, NAP-2, NT-3, TNF-b and Thrompoietin) by ELISA. The CXCL13 level was found to be higher (2.75 fold) in MG patients ($N=25$) than in the control subjects ($N=10$) (data not shown) and the expression of CXCL13 in thymic tissues and serum CXCL13 level were characterized in more detail.

3.1. Serum CXCL13 level is associated with disease severity in MG

To increase the sample size, sera from a total of 194 cases (male: 65 and female: 129) with MG and 233 control subjects (male: 86 and female: 147) were recruited in our study with gender and age matched between the two groups (Table 1). Although mean serum CXCL13 level was similar (Case: 107.55 pg/ml and Control: 105.28 pg/ml), the serum CXCL13 level was found to be different in various categories of MG patients. Interestingly, serum CXCL13 level was found to be associated with gender ($p=0.004$; as calculated by the GLM test) and age ($p=0.013$; as calculated by correlation analysis). However, in the control group gender and age were not associated with serum CXCL13 level. Therefore, the difference of serum CXCL13 level between the case and control samples might be due to the age and gender effects. When adjusted for gender and age, onset class (early–onset: below 40 years, and late–onset: above 40 years) and clinical stage (stage I for ocular type and stage II/III for general type) were also shown to be associated with serum CXCL13 level in MG (Table 2). Among the 194 cases, 60% (118/194) have thymic lymphoid hyperplasia. If we only examined the female cases with lymphoid hyperplasia, high CXCL13 level was significantly associated with clinical stages II and III (Table 3). Consistent with the clinical findings, patients who had undergone prednisolone treatment had higher CXCL13 than those who had not ($p=0.025$), suggesting that CXCL13 is correlated to the disease severity.

Table 2
Serum CXCL13 and different clinical features of myasthenia gravis.

Features		*N*	Mean CXCL13 (pg/ml)	*p* value*	*p* value**
Onset class	Early–onset	117	130.66	0.003	0.016
	Late–onset	77	72.44		
Clinical stage	Stage I	71	83.18	0.074	0.017
	Stage II	108	111.28		
	Stage III	15	196.00		
	Ocular type	71	83.18	0.076	0.028
	General type	113	121.61		
Anti-AchR	Positive	154	110.32	0.828	0.413
	Negative	40	96.86		
Immunotherapy (Prednisolone)	Yes	81	134.46	0.025	0.025
	No	113	88.26		
Thymectomy	Yes	91	111.41	0.871	0.701
	No	103	104.14		
Thymus status	Atrophy	37	83.88	0.263	0.726
	Hyperplasia	118	111.97		
	Thymoma	39	116.62		

* *p* Value was calculated by GLM method.
** *p* Value was calculated by GLM method with gender and age adjustment.

Table 3
Serum CXCL13 in myasthenia gravis patients with lymphoid hyperplasia.

Features		Female				Male			
		Early–onset		Late–onset		Early–onset		Late-onset	
		N	Mean CXCL13 (pg/ml)	*N*	Mean CXCL13 (pg/ml)	*N*	Mean CXCL13 (pg/ml)	*N*	Mean CXCL13 (pg/ml)
Clinical stage	Stage I	30	121.93	6	73.83	10	48.70	2	27.50
	Stage II	34	158.18	16	84.38	10	67.60	5	72.20
	Stage III	2	235.00	2	135.00	1	65.00	0	-
Anti-AchR	Positive	48	158.35	20	87.50	18	53.50	6	59.33
	Negative	18	105.83	4	78.25	3	83.33	1	60.00
Immunotherapy (Prednisolone)	Yes	30	160.47	11	92.64	3	48.33	2	27.50
	No	36	130.33	13	80.31	18	60.17	5	72.20
Thymectomy	Yes	28	123.29	12	108.25	10	59.10	3	81.00
	No	38	159.32	12	63.67	11	57.91	4	43.25

3.2. CXCL13 expression in MG thymic tissues

Two major histology types were observed in thymus tissues obtained from thymectomy for MG: lymphoid hyperplasia and thymoma. Since CXCL13 and its receptor CXCR5 have been reported to regulate the compartmentalization of specific lymphoid cell types (Förster et al., 1996; Gunn et al., 1998; Ansel et al., 2000), we examined the MG thymic tissues by immunohistochemical staining and correlated CXCL13 expression with the histology types. As shown in Fig. 1, CXCL13 and CXCR5 were preferentially expressed in the follicles of hyperplasia and their surrounding areas. By contrast, the thymoma tissues were not reactive with CXCL13 or CXCR5. To confirm the high-level CXCL13 expression in MG patients with lymphoid hyperplasia, we performed quantitative RT-PCR analysis of the two genes. As shown in Fig. 2A and 2B, except for sample #13, CXCL13 and CXCR5 transcript levels were significantly higher in thymic lymphoid hyperplasia tissues than in thymoma tissues. Generally speaking, CXCR5 transcript level paralleled that of CXCL13 in MG thymus tissues.

4. Discussion

In the current study, we have taken a systematic approach to investigate serum cytokines and chemokines in MG. Our data indicate that serum CXCL13 is elevated in some MG patients with various thymic histology types. By contrast, mRNA and protein expressions of CXCL13 and its cognate receptor, CXCR5, were much higher in thymic tissues with lymphoid hyperplasia. Although the mean serum CXCL13 level in MG patients was comparable between the thymoma group and the thymic lymphoid hyperplasia group, two out of 39 thymoma patients (5%) has outstandingly high CXCL13 (460 and 1125 pg/ml), compared to the other 37 patients, whose levels were all below 200 pg/ml (100 pg/ml in average). On the other hand, 24 out of 118 MG patients (20%) with thymic lymphoid hyperplasia have serum CXCL13 level higher than 200 pg/ml ($p<0.05$; as calculated by chi-square test). Thus, CXCL13, as a B cell chemoattractant, was significantly associated with thymic lymphoid hyperplasia (Fig. 2), and high serum CXCL13 level was indicative of severe disease. In this sense, serum CXCL13 can be regarded as a marker of MG disease activity, especially in patients with thymic lymphoid hyperplasia. While this work was in progress, Meraouna et al. (2006) reported that CXCL13 expression was increased in the thymus as well as in sera of patients, who did not undergo glucocorticoid therapy and that the CXCL13 level decreased with glucocorticoid treatment, in correlation with clinical improvement. Their data have indicated that CXCL13

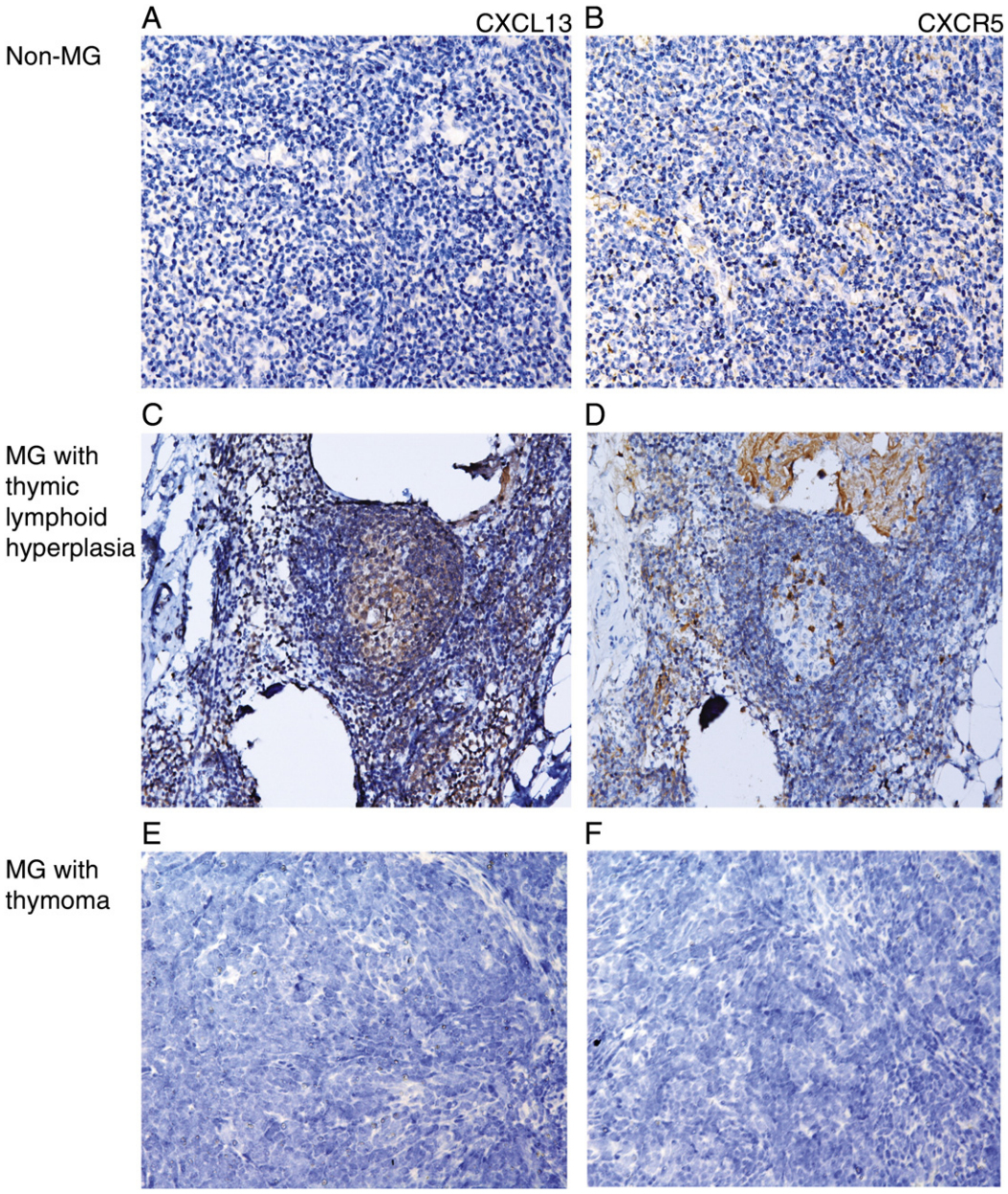

Fig. 1. Immunohistochemical staining of thymic tissues. Immunohistochemical stain of CXCL13 and CXCR5 on thymic tissues from different clinical backgrounds. The results show increased expression of CXCL13 and CXCR5 in MG patients with thymic lymphoid hyperplasia. A and B: thymus of non-MG individual. C and D: thymus of MG patients with lymphoid hyperplasia. E and F: thymoma of MG patients. A representative result from five samples of MG with thymic lymphoid hyperplasia and the other from five thymoma samples are presented here. Original magnification 200×.

could be considered as a therapeutic target for autoimmune diseases characterized by ectopic lymphoid germinal center formation, including MG (Meraouna et al., 2006). Note that in their study CXCL13 was selected originally through microarray transcriptome analysis (Le Panse et al., 2006), while our conclusion was drawn from serum cytokine and chemokine analysis. Taken together, our data indicate that serum CXCL13 reflects the general status of MG severity and there is heterogeneity in MG patients regarding etiology and immune and inflammatory response. However, there is compounding biological factors, such as gender and treatment response. As shown in Table 4, the serum CXCL13 levels are different for MG patients between the age group of younger than 40 and the age group of 40 and above. Moreover, as a whole, the serum CXCL13 levels are different between MG patients and the age-matched controls. Of significance, the male patients of 40 years and above have lower CXCL13 (66.52 pg/ml) compared to the control (95.90 pg/ml) ($p<0.001$), and the female patients of 40 years and above showed the same trend ($p<0.01$). We consider that female young patients receiving prednisolone probably represent a unique subgroup of MG, and they tend to be more severe in the clinical course. On the other hand, the males who present late at onset might have a protracted course prior to the diagnosis. Thus, our data provide a broad picture of the distribution of serum CXCL13 level in MG patients and its clinical correlation, such as disease severity. Future perspective studies are warranted to demonstrate the clinical utility of serum CXCL13 in MG.

CXCL13 has been reported to be required for B1 cell homing, natural antibody production, and body cavity immunity (Ansel et al., 2002). B1 cells have been shown to possess autoantigen presenting capability and they can be preferentially attracted to the site of CXCL13 expression (Sato et al., 2004). The ectopically expressed CXCL13 in the thymus of MG patients with lymphoid hyperplasia may indicate that B1 cells can be recruited to the thymus. We have also

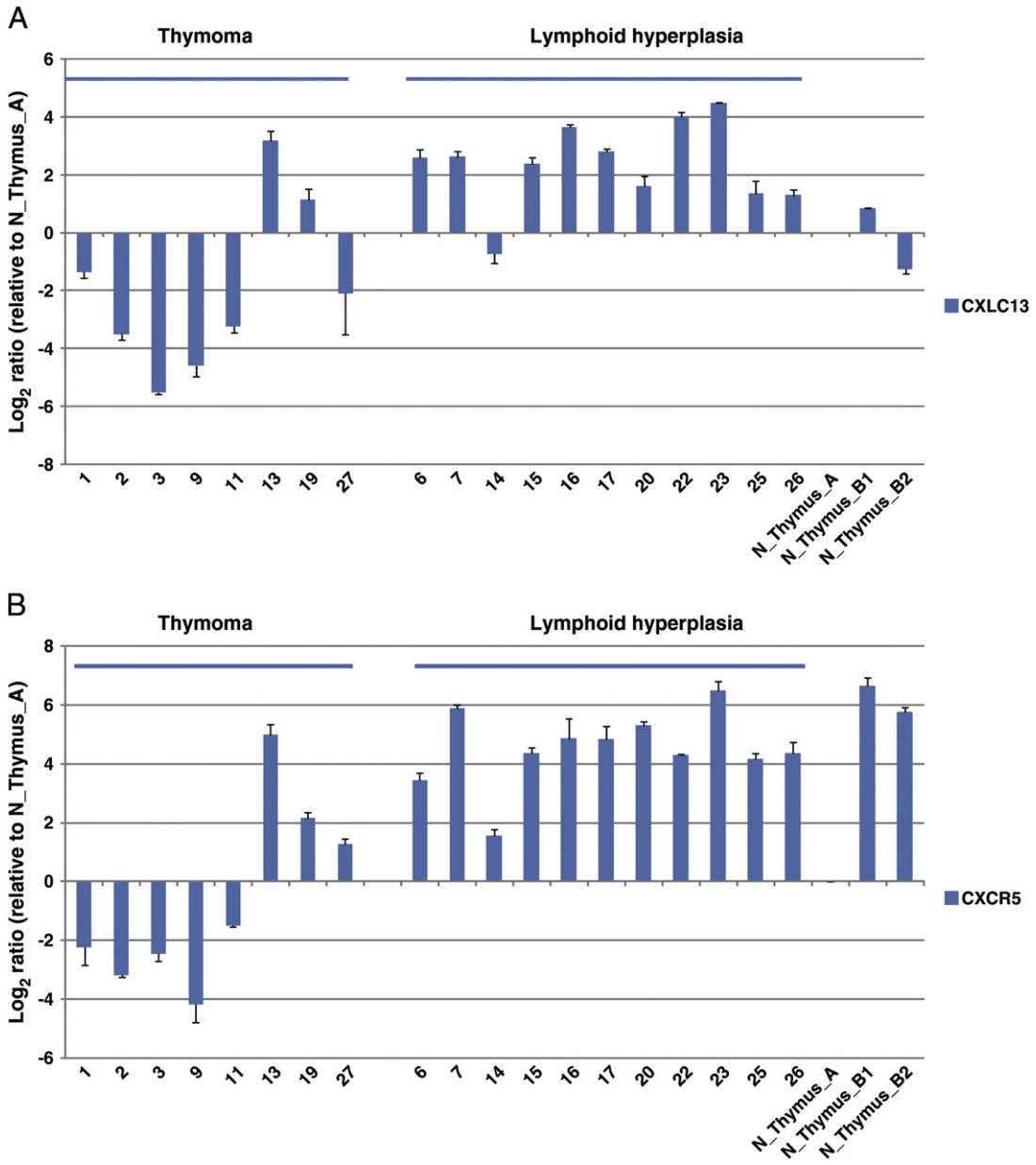

Fig. 2. mRNA expression in thymic tissues. Quantitative analysis of (A) CXCL13 and (B) CXCR5 mRNA expression in thymic tissues from MG (both lymphoid hyperplasia and thymoma) and normal control (N_thymus_A, N_Thymus_B1 and N_Thymus B2) were performed by TaqMan Gene Expression Assay. Expression level of each sample was shown relative to normal thymus (N_thymus_A). The results were averaged from three independent experiments.

found that the level of CXCR5 expression is enhanced in lymphoid hyperplasia cases. There appears to be a good correlation for CXCL13 and CXCR5 expressions in the thymus, indicating that CXCL13 can modulate local immune reactions by recruiting specific cell types to the thymus. The exact identities of these cells, however, need to be further characterized, as the current data can not exclude the possibility that other cell types than B1 are also recruited to the thymus by CXCL13.

Table 4
Serum CXCL13 in different subgroups of cases and controls.

	Case ($N=194$)			Control ($N=233$)		
	Male	Female	Total	Male	Female	Total
Patient number (Age <40 years)	23	52	75	14	45	59
Mean CXCL13 (pg/ml)	107.83	150.62	137.49	85.36	112.11	105.76
Patient number (Age≧40 years)	42	77	119	72	102	174
Mean CXCL13 (pg/ml)	66.52	100.76	88.68	95.90	111.61	105.11

In thymus with lymphoid hyperplasia, CXCL13 has been found to be preferentially expressed by the epithelial cells (Meraouna et al., 2006). It is intriguing to understand the control mechanism for CXCL13 expression and what trigger ectopic and high CXCL13 expression in the thymus, as it can be of relevance to the development of specific treatment for CXCL13-overexpressing MG patients. CXCL13 is normally expressed in secondary lymphoid organs, and members of the lymphotoxin/tumor necrosis factor (LT/TNF) family play important roles in controlling the expression of lymphoid chemokines (Ngo et al., 1999). On the other hand, aberrant CXCL13 expression has been associated with chronic inflammatory diseases, such as Sjögren's syndrome and rheumatoid arthritis (Barone et al., 2005; Shi et al., 2001). Of relevant interest, CXCL13 production has recently been

linked to the activation of NF-κB pathway through NF-κB-inducing kinase (NIK) in Nod2-dependent signaling (Pan et al., 2006).

In MG, there appears to be a wide variation of the CXCL13 expression in the thymus as well as of the serum CXCL13 level. In the thymoma tissue, the histology and gene expression are distinctly different from those in the thymic lymphoid hyperplasia. However, we observed that a few thymoma tissues did show overall gene expression patterns similar to those of the lymphoid hyperplasia (Shiao and Tsai, unpublished), suggesting that there might be an overlap between the two groups. It would be of clinical significance, besides histological classification, to identify thymoma cases with high CXCL13 expression, as a different group for selecting alternative treatment options. To implement therapeutics tailored to individual difference, additional markers might also need to be included. On the basis of the present study and the conclusion drawn by Le Panse et al. (2006) we think that a combination of serum protein profiling and thymus transcription analysis have a potential to yield improved protocols for classifying MG patients for individualized treatment.

Disclosure

The authors report no conflicts of interest.

Acknowledgements

The authors wish to thank Drs. Fang Liao and Jih-Shyun Su for critically reading and insightful discussion of this manuscript. We are also grateful to the members of the Myasthenia Gravis Club in the Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan for participating in this study. This work was supported by intramural funds from the National Health Research Institutes (to SFT).

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