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Association between acetylcholine receptor characteristics in biceps motor endplates and the epidemiological predictors for conversion from ocular to generalized myasthenia gravis

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Objective: Epidemiological studies have identified various predictors of conversion from ocular myasthenia gravis (OMG) to secondary generalized myasthenia gravis (SGMG), but none have been confirmed. We investigated the effects of the epidemiological conversion predictors on the destruction of motor endplates in the biceps of patients with OMG and attempted to identify predictors of conversion to SGMG histologically.

Methods: Patients with clinically diagnosed OMG who requested immunohistological diagnosis and who underwent muscle biopsy were included in this study. We immunostained the biceps motor endplate and semi-quantitatively measured the density and number of AChRs to determine their association with the epidemiological predictors of conversion from OMG to SGMG.

Results: Thirteen patients with OMG were included, of which two patients with positive AChR antibody and concomitant thymoma converted to SGMG. In the classification according to the presence of AChR antibody, the AChR densities tended to be lower in the antibody-positive group than in the negative group (p=0.079), and the AChR numbers were significantly lesser in the AChR antibody-positive group than in the negative group (p=0.019). There were no differences in AChR densities or numbers according to sex, presence of thymic abnormalities, or presence of comorbid autoimmune diseases.

Conclusion: In OMG, the AChR numbers in motor endplates of the biceps were significantly lesser in the AChR antibody-positive group than in the negative group. Since the muscle strength tends to decrease as the number of AChRs decreases, AChR antibody positivity may be a predictor of OMG to SGMG conversion, but further studies are needed to confirm.

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Key words: ocular myasthenia gravis, secondary generalized myasthenia gravis, acetylcholine receptor antibody, motor endplate

Introduction

Myasthenia gravis (MG) is an autoimmune disease that manifests as skeletal muscle fatigue due to the development of autoantibodies against several target antigens on the postsynaptic membrane of the neuromuscular junction. Approximately 50% of patients with MG develop ocular myasthenia gravis (OMG) with symptoms of ptosis and diplopia only[1-6], and in approximately 10–85% of patients with OMG, the disease converts to secondary generalized myasthenia gravis (SGMG) that manifests as limb muscle weakness, bulbar symptoms, and dyspnea[1-17]. Epidemiological studies have identified acetylcholine receptor (AChR) antibody positivity as a predictor of conversion from OMG to SGMG[2-4, 7-12]. Other predictors include disease onset in the old age[6, 16], female sex[9, 13], positive results of trapezius repetitive nerve stimula-

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tion test (RNST)[3, 8], presence of thymic abnormalities[1-3, 8, 12-15], and presence of comorbid autoimmune diseases[2]. However, none of these epidemiological predictors have been confirmed, and the indicators for immunosuppressive therapy in the prevention of conversion from OMG to SGMG are unclear.

The diagnosis of OMG is often difficult, even for experienced neurologists, because symptoms such as ptosis and eye movement disorders are not MG-specific, the detection rate of pathogenic autoantibodies and RNST abnormalities is low[18], and results of intravenous edrophonium test can be positive for diseases other than MG[19]. Tsujihata et al. immunostained the motor endplates on the biceps brachii muscle in patients with OMG and reported the deposition of complement C3, destruction of the motor endplates, and a significant reduction in the optical densities (ODs) of the AChRs[20]. Although muscle biopsy is not used frequently to assess the neuromuscular junction, it is useful for the histological diagnosis of MG and, especially, for the diagnosis of MG that is difficult to diagnose using conventional methods. However, the pathological effects of the aforementioned epidemiological predictors on the skeletal muscles of the limbs of patients with OMG have not been investigated.

In this study, we measured the densities and numbers of the AChRs in the motor endplates of the biceps brachii in patients with OMG and investigated their association with various epidemiological predictors for conversion to SGMG. Our study provides histological insights to identify predictors of conversion from OMG to SGMG.

Materials and Methods

Participant enrollment and data collection

We performed the muscle biopsies of the biceps brachii in patients who were clinically diagnosed with OMG and who requested immunohistochemical diagnosis at Nagasaki University Hospital between October 1996 and November 2016. OMG is diagnosed clinically based on the presence of at least one positive result of the results for the presence of the AChR or muscle-specific receptor tyrosine kinase (MuSK) antibody, intravenous edrophonium test, or RNST at 3 Hz in patients with symptoms of ptosis, eye movement disorders, or diplopia. The levels of the AChR and MuSK antibodies were measured using radioimmunoassay. The RNST was judged positive when the attenuation rate of the amplitude of the 4th or 5th stimulus was 10% or more of the amplitude of the 1st stimulus. Thymic abnormality was diagnosed based on computed tomography and/or magnetic resonance imaging findings or pathology if thymectomy was performed. To prevent the possible inclusion of patients with generalized MG in the study, patients who converted to SGMG within 1 month of muscle biopsy were excluded. The clinical grade of disease severity in patients was determined according to the Myasthenia Gravis Foundation of America clinical classification[21]. The medical records of all patients were reviewed retrospectively.

All patients provided informed consent, and the study was approved by the Human Ethics Review Committee of the Nagasaki University Hospital (approval number: 21111507).

Histochemical- and immuno-staining

Biopsy cryosections were stained using hematoxylin and eosin, the modified Gomori trichrome stain, and various other enzymes for differential diagnosis. Immunostaining for observing the AChRs and deposits of complement C3 under a light microscope was performed using peroxidase (grade III; Toyobo Chemical, Osaka, Japan)-labeled α-bungarotoxin (Miami Serpentarium, Miami, FL) and peroxidase-labeled rabbit human complement C3 (Dakopatts, Glostrup, Denmark), respectively, as described previously[22].

Semi-quantitative analysis of motor endplates

To analyze the motor endplates of the biceps brachii, we identified the sites that react with peroxidase-labeled α-bungarotoxin, as it binds quantitatively with the human AChRs. Approximately 10 endplates were analyzed for each patient. Briefly, using the vector 'Colour 2 of H DAB' in the Colour Deconvolution plugin with NIH imageJ2, the mean OD of a straight line on the endplates (AChR densities) and the integrated density in the rectangle around the endplates (AChR numbers) were derived from each endplate, after subtracting the background OD values in the sarcoplasm close to the same endplates (Figure 1). Strictly speaking, the "numbers" represented the sum of the OD values of the labeled AChRs inside the rectangle, which indirectly reflect the number of AChRs per endplate. The ODs were calculated using the uncalibrated OD function of NIH imageJ2. All measurements were performed blinded to clinical data.

Statistical analysis

The data are presented as the median (interquartile range) value. Categorical variables were analyzed using Fisher exact test and continuous variables were analyzed with Wilcoxon rank-sum test. Statistical significance was set at $p \leq 0.05$.

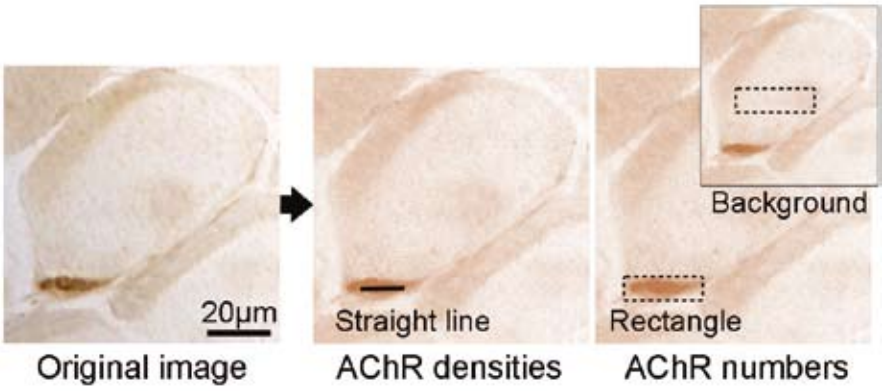

Figure 1 Semi-quantitative analysis of the motor endplates.
Analysis of the sites that react with peroxidase-labeled α -bungarotoxin. Derivation of the mean OD of a straight line on the endplates (AChR densities) and the integrated density in a rectangle around the endplates (AChR numbers) from each endplate, after subtracting the background OD values in the sarcoplasm close to the same endplates.
OD: optical density, AChR: acetylcholine receptor

Results

During the study period, muscle biopsies were performed on 14 patients clinically diagnosed with OMG. However, 13 patients were included in the analysis because one patient converted to SGMG within 1 month of the biopsy. The clinical features of the 13 patients are shown in Table 1. The median age of onset was 62 (interquartile range: 45–71) years, with 6 males (46.2%) and 7 females (53.8%). The median time from onset to muscle biopsy was 4 (interquartile range: 2–16) months. Eight patients showed positive AChR antibody test results (61.5%), and no patient showed positive MuSK antibody test results. The follow-up periods after the biopsy for patients nos. 7 and 12 were 1 and 5 months, respectively, while those for the others were more than 4 years. Patients nos. 8 and 9, both showed positive AChR antibody test results and had thymoma, converted from OMG to SGMG 12 and 57 months after the onset, respectively.

Immunostaining showed C3 deposition in all patients. The OD measurements were analyzed for each epidemiological predictor for the conversion from OMG to SGMG. In the classification according to the presence of the AChR antibody (Figure 2), the AChR densities tended to be lower in the AChR antibody-positive group than in the AChR antibody-negative group (p=0.079), and the AChR numbers were significantly decreased in the AChR antibody-positive group than in the AChR antibody-negative group (p=0.019; Table 2). In a sub-analysis of study participants who did not undergo immunosuppressive therapy before muscle biopsy, AChR densities were not statistically different between the AChR antibody-positive and negative groups (p=0.2012), whereas AChR numbers were significantly lower in the AChR antibody-positive group than in the negative group (p=0.045). There were no significant differences in AChR densities or numbers according to sex (p=0.568, p=0.153, respectively) or presence of thymic abnormalities (p=0.643, p=0.643, respectively) or comorbid autoimmune diseases (p=0.165, p=0.280, respectively; Table 3).

Discussion

The primary pathogenic mechanism of AChR antibody-positive MG is complement-mediated destruction of the motor endplates and a decrease in the number of AChRs. The motor endplate destruction of the biceps brachii is manifested in both OMG and generalized MG, but the degree of destruction is milder in OMG than in generalized MG[23]. In this study, we demonstrated that the number of AChRs in the motor endplates of the biceps brachii in OMG was lesser in the AChR antibody-positive group than in the AChR antibody-negative group. Since the muscle strength tends to decrease as the number of AChRs on the motor endplate decreases[22, 24], we speculate that this result provides histological corroboration that AChR antibody-positive OMG is more likely to convert to SGMG than negative OMG. However, there was no significant difference in the conversion rate from OMG to SGMG between the AChR-positive and negative groups (Table 2), even though 2 of 13 study participants (15.4%) converted to SGMG were AChR antibody-positive. Since

the use of immunosuppressants for patients with OMG not only reduces antibody titers and improves symptoms[25], but also prevents conversion to SGMG with a conversion rate of about 7-17%[1, 10, 13, 16], the non-significant statistical difference observed in this study is inevitable, as most of the study participants had undergone immunosuppressive therapy after the biopsy. There were no statistical differences in the densities and numbers of AChRs in the motor endplates according to other epidemiological predictors for conversion from OMG to SGMG, such as sex and presence of thymic abnormalities and comorbid autoimmune diseases (Table 3).

Although no treatment guidelines exist for OMG, acetylcholinesterase inhibitors are used as first-line treatment for patients with OMG. However, since acetylcholinesterase inhibitors can help alleviate symptoms but rarely leads to complete resolution, patients with an unsatisfactory response to acetylcholinesterase inhibitors are candidates for immunosuppressive therapy. Corticosteroids are used as the initial immunosuppressive agent, and steroid-sparing agents, such as tacrolimus, azathioprine, and mycophenolate mofetil are frequently used in long-term therapy. Furthermore, recent studies have shown that treatment with intravenous methylprednisolone is associated with faster symptom improvement[26, 27]. Immunosuppressive therapy interferes with inflammation, in particular, steroids increase the number of AChRs[10, 17]. Interestingly, in retrospective studies of patients with AChR antibody-positive OMG, Apinyawasisuk et al.[13] and Mee et al.[28] reported that immunosuppressive therapy can prevent this subset of patients from converting to SGMG. Given that AChR antibody positivity is a predictor of conversion to SGMG, it may be one of the indicators to consider in choosing immunosuppressive therapy for patients with OMG.

The median age of the participants in this study was 62

Table 1 Clinical features of patients with ocular myasthenia gravis

Patient No.	Sex	Age of onset (years)	Duration from onset to biopsy (months)	AChR antibody (nmol/L)	Repetitive nerve stimulation test results	Thymic abnormality	Comorbid autoimmune disease	Pre-biopsy therapy	Post-biopsy therapy	Follow-up period (months)	Highest MGFA grade
1	M	71	36	Negative	Negative	None	None	None	IVMP, PSL, tacrolimus	81	I
2	M	27	54	1.9	Positive (trapezius)	Hyperplasia	Basedow's disease	None	AChEI, Thymectomy	156	I
3	M	68	1	Negative	Positive (facial, trapezius)	None	None	AChEI	IVMP, PSL	181	I
4	M	71	9	Negative	Negative	None	None	None	IVMP, PSL	113	I
5	F	64	2	46	Negative	None	None	AChEI	AChEI	73	I
6	F	41	4	220	Negative	None	SLE, Chronic thyroiditis	PSL, AChEI	PSL, AChEI	48	I
7	M	49	22	Negative	Negative	None	None	None	AChEI	1	I
8	F	75	1	4.4	Negative	Thymoma	None	None	PSL, AChEI	83	V
9	F	53	2	14.2	Not available	Thymoma	None	None	Thymectomy, Chemoradiation therapy	153	V
10	M	62	10	83.4	Negative	None	SLE	PSL	IVMP, PSL	263	I
11	F	36	5	9.5	Negative	Hyperplasia	None	None	PSL	137	I
12	F	81	4	3.8	Negative	None	Chronic thyroiditis	None	PSL, AChEI	5	I
13	F	57	2	Negative	Negative	None	None	None	IVMP	275	I

AChR: acetylcholine receptor, MGFA: Myasthenia Gravis Foundation of America, M: male, F: female, SLE: systemic lupus erythematosus, AChEI: acetylcholinesterase inhibitor, PSL: prednisolone, IVMP: intravenous methylprednisolone

(interquartile range: 45–71) years, the proportion of females was 53.8%, and the complication rate of autoimmune diseases was 30.8%, which is consistent with the results of previous epidemiological studies on OMG, which were median age (46-75.9 years)[2, 5-8, 10, 11, 13, 14, 17], the proportion of females (27-72.2%)[1-8, 10, 11, 13-17], and complication rate of autoimmune diseases (15.1-32.5%)[2, 4, 6, 13]. In our study, the AChR antibody positivity rate was 61.5%, the thymoma complication rate was 15.4%, and the thymic hyperplasia complication rate was 15.4%, which is consistent with the findings of previous studies, which were AChR antibody positivity rate (35-76.7%)[1-5, 7, 8, 10, 11, 14, 17], thymoma complication rate (2-25%)[1, 3, 5, 6-8, 10, 15, 16], and thymic hyperplasia complication rate (3.5-55%)[1-3, 6, 16]. The positivity rate of the trapezius RNST has been reported as 23.5% to 46%[3, 8, 16], but it was considerably low (17%) in this study. Classified according to the presence of the AChR antibodies (Table 2), all patients with thymic abnormalities and those with comorbid autoimmune diseases (3 of 4 patients had thyroid disease) were AChR antibody positive. This result is not surprising as AChR antibody-positive patients with MG are more likely to have thymic abnormalities and thyroid disease[29].

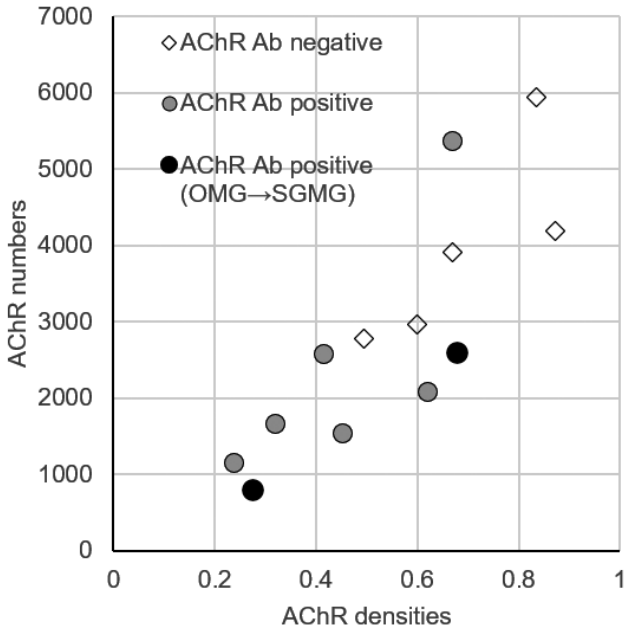

Figure 2 The densities and numbers of AChRs in patients with OMG.
In the classification according to the presence of the AChR Ab, lower mean AChR densities in the Ab-positive group than in the negative group (p=0.079), and significantly lesser mean number of AChRs in the AChR Ab-positive group than in the negative group (p=0.019). Two patients who converted to SGMG after muscle biopsy were AChR Ab positive, but did not have lower densities or numbers of AChRs than other AChR Ab positive patients.
The densities and numbers of AChRs are expressed as optical density values.
AChR: acetylcholine receptor, OMG, ocular myasthenia gravis; Ab: antibody, SGMG: secondary generalized myasthenia gravis

The pathogenesis of MG in patients of the AChR antibody-negative group in this study was complement-mediated neuromuscular junction destruction, as complement C3 deposition was observed in all patients, and no study participant had congenital myasthenia. Although the levels of low-density lipoprotein receptor-related protein 4 (LRP4) antibody was not measured in this study, complement deposition on the motor endplates has not been demonstrated in human LRP4 antibody-positive patients with MG. All patients were negative for the MuSK antibody. It is possible that patients in the AChR antibody-negative group in this study had AChR antibodies below the level of detection sensitivity. Patient no. 4 did develop AChR antibodies more than 2 years after the biopsy.

The limitations of this study must be considered. First, because we did not include all patients with OMG who visited our hospital, but only those who requested muscle biopsy for immunohistochemical diagnosis, the sample size was small and some selection bias was inevitable. Second, the two patients who converted to SGMG during the follow-up period were AChR antibody-positive and both had thymoma. However, due to the small sample size, neither could we analyze thymoma complication as an independent factor for conversion to SGMG, nor could we perform multivariate analysis.

To conclude, we demonstrated that in OMG, the number of AChRs on the motor endplates on the biceps brachii was significantly lesser in the AChR antibody-positive group than in the AChR antibody-negative group. AChR antibody positivity may be a predictor of OMG to SGMG conversion, but further studies are needed to confirm this finding.

Declaration of interest: None

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Table 2 Comparison of clinical features and AChR density/number between AChR antibody-positive and negative patients

AChR antibody	Positive (n=8)	Negative (n=5)	*p* value
Female	6 (75.0)	1 (20.0)	0.1026
Age at onset, years	57.5 [37.3-72.3]	68.0 [53.0-71.0]	0.464
Duration from onset to biopsy, months	4.0 [2.0-8.8]	9.0 [1.5-29.0]	0.712
RNST abnormalities	1 (12.5)	1 (20.0)	1.0000
Thymoma	2 (25.0)	0	0.4872
Thymic hyperplasia	2 (25.0)	0	0.4872
Comorbid autoimmune diseases	4 (50.0)	0	0.1049
Conversion to SGMG	2 (25.0)	0	0.4872
AChR densities	0.43 [0.29-0.66]	0.67 [0.55-0.85]	0.079
AChR numbers	1875 [1248-2595]	3907 [2868-5067]	0.019

Data are presented as n (%) or median [interquartile range] values.
AChR densities and numbers are expressed as optical density values.
AChR: acetylcholine receptor, RNST: repetitive nerve stimulation test, SGMG: secondary generalized myasthenia gravis

Table 3 Comparison of AChR density/number for each predictor of secondary generalized myasthenia gravis conversion.

Sex	Male (n=6)	Female (n=7)	*p* value
AChR densities	0.63 [0.43-0.72]	0.45 [0.32-0.68]	0.568
AChR numbers	3437 [2366-4489]	2082 [1531-2601]	0.153
Thymic abnormalities	With (n=4)	Without (n=9)	
AChR densities	0.64 [0.36-0.68]	0.49 [0.37-0.75]	0.643
AChR numbers	2342 [1115-4676]	2770 [1599-4051]	0.643
Comorbid autoimmune diseases	With (n=4)	Without (n=9)	
AChR densities	0.39 [0.26-0.61]	0.62 [0.45-0.76]	0.165
AChR numbers	1599 [1248-4442]	2770 [2329-4051]	0.280

Data are presented as median [interquartile range] values.
AChR densities and numbers are expressed as optical density values.
AChR: acetylcholine receptor

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