Journal of the Neurological Sciences 443 (2022) 120494

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

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

Motor end-plate analysis to diagnose immune-mediated myasthenia gravis in seronegative patients☆

Atsushi Nagaoka [a,*], Akira Tsujino [a], Hirokazu Shiraishi [b], Tadashi Kanamoto [b], Tomoaki Shima [b], Shunsuke Yoshimura [b], Teiichiro Miyazaki [b], Yohei Tateishi [b], Mitsuhiro Tsujihata [c], Masakatsu Motomura [d], Susan Maxwell [e], Osamu Higuchi [f], David Beeson [e], Angela Vincent [e]

[a] Department of Clinical Neuroscience, Unit of Clinical Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki City, Japan
[b] Department of Neurology and Strokology, Nagasaki University Hospital, Nagasaki City, Japan
[c] Nagasaki Kita Hospital, Nagasaki City, Japan
[d] Medical Electronic Course, Nagasaki Institute of Applied Science, Nagasaki City, Japan
[e] Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford, UK
[f] Department of Neurology, Nagasaki Kawatana Medical Center, Nagasaki, Japan

ARTICLE INFO

Keywords:
Myasthenia gravis
Motor end-plate
Complement
Clustered AChR antibody
Seronegative

ABSTRACT

This study aimed to evaluate the diagnostic usefulness of motor end-plate (MEP) analysis along with clustered acetylcholine receptor (AChR) antibody (Ab) assays in patients with myasthenia-like symptoms but negative routine AChR and muscle-specific kinase (MuSK) Ab tests. MEP analysis of muscle biopsies of the biceps brachii was performed in 20 patients to try to differentiate between those with or without immune-mediated myasthenia gravis (MG). Using a quantitative method, complement C3 deposition and AChR densities in MEPs were examined. Independently, cell-based assays were used to detect serum clustered-AChR Abs. Only five of 20 patients had complement deposition at MEPs; four of these patients had reduced AChR densities similar to those in patients with typical AChR Ab positive MG, and distinct from those in the remaining 15 patients. Two of the four serum samples from these patients had clustered-AChR Abs. All complement-positive patients were considered as having immune-mediated MG and improved with appropriate treatments; although one patient presented with MG 3 years later, the remaining patients had other diagnoses during over 10 years of follow-up. These results suggest the usefulness of MEP analysis of muscle biopsies in diagnosing immune-mediated MG in seronegative patients with myasthenia-like symptoms but, due to the invasiveness of the muscle biopsy procedure, clustered AChR Abs should, if possible, be tested first.

1. Introduction

The definitive diagnosis of MG can be hard to make when patients complaining of myasthenia-like symptoms are seronegative for routine AChR and MuSK antibody tests, show no significant waning phenomenon with repetitive nerve stimulation and equivocal responses to the intravenous edrophonium (Tensilon) test. Particularly in ocular MG, about 50% of patients are negative for routine antibodies, and about 70% show no waning phenomenon on repetitive nerve stimulation [1]. Tensilon tests are positive in about 90% of cases [2], but false-positive responses can confound the diagnosis [3,4,6,7]. Mitochondrial myopathy [4], ophthalmopharyngeal muscular dystrophy [5], blepharospasm [6], and Miller-Fisher syndrome [7] can each be misdiagnosed as ocular MG. Although in ocular MG only the extraocular muscles are affected,

☆ This work is dedicated to the memory of Professor Toshiro Yoshimura, who passed away on September 16, 2014.

* Corresponding author at: Department of Clinical Neuroscience, Unit of Clinical Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

E-mail addresses: a-naga@nagasaki-u.ac.jp, akrtjn@nagasaki-u.ac.jp (A. Nagaoka), akrtjn@nagasaki-u.ac.jp (A. Tsujino), tomoakishima0915@nagasaki-u.ac.jp (T. Shima), shun-yoshimura@nagasaki-u.ac.jp (S. Yoshimura), tmiyazaki@nagasaki-u.ac.jp (T. Miyazaki), ytate@nagasaki-u.ac.jp (Y. Tateishi), kita_m_tsuji@mx22.tiki.ne.jp (M. Tsujihata), MOTOMURA_Masakatsu@NiAS.ac.jp (M. Motomura), susan.maxwell@ndcn.ox.ac.uk (S. Maxwell), osmhgc_nkmc@nagasaki-u.ac.jp (O. Higuchi), David.beeson@ndcn.ox.ac.uk (D. Beeson), angela.vincent@ndcn.ox.ac.uk (A. Vincent).

https://doi.org/10.1016/j.jns.2022.120494
Received 14 June 2022; Received in revised form 8 November 2022; Accepted 10 November 2022
Available online 14 November 2022

the symptoms can be a serious burden on the patient's daily and social activities [8] and a correct diagnosis is essential.

Approximately 85% of Japanese patients with MG have AChR Abs and up to 5% have MuSK Abs, as measured by radioimmunoassays (RIAs) [9], leaving around 10% without a definitive serological diagnosis. We hypothesized that if a patient had MG, complement deposition and reduced AChR densities at the MEP would be found, as previously reported for AChR Ab positive MG patients [10,11]. Therefore, we performed the same measurements in patients for whom a definitive diagnosis remained undetermined. In addition, we looked for antibodies that bind only to AChR when clustered on the cell surface as found at the NMJ; these clustered-AChR Abs can be detected in up to 50% of patients negative on routine tests, but are not yet widely available [12]. To establish the specificity of our findings we observed the clinical course of all previously seronegative patients to document their final diagnoses.

2. Material and methods

2.1. Subjects

Myasthenia-like symptoms were defined as those involving at least ocular muscle weakness (such as ptosis or diplopia) concurrent with muscular fatigue. Muscle biopsies were discussed with patients who had myasthenia-like symptoms, were seronegative for routine AChR and MuSK antibody tests (RIA assays), and had <10% of the waning phenomenon with repetitive nerve stimulation. When enrolling patients under the code of ethics at the time, the attending doctors explained that muscle biopsies were performed to diagnose or confirm the diagnosis of MG, and in those cases that were not confirmed, to look for evidence of other neuromuscular diseases such as mitochondrial encephalomyopathy or oculopharyngeal muscular dystrophy. They also made it clear that using muscle biopsy, which is invasive, as a diagnostic test for MG was based on our previous research findings [11,14,16] and was not an internationally established test. These explanations have been performed in the same manner since 1989. For this study, we obtained new approval for the study of muscle biopsy in patients with potential misdiagnosis of myasthenia from the Ethics Committee of Nagasaki University Hospital. We enrolled twenty patients with muscle biopsies undertaken in our hospital between 1994 and 2015 and examined their medical records up to 2020. The clinical grade of disease severity was evaluated according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification system. This study was approved by the Human Ethics Review Committee of Nagasaki University Hospital.

2.2. Muscle biopsies

Cryosections from the biceps brachii were stained with hematoxylin and eosin, modified Gömöri trichrome stain, and various enzymes for different diagnoses. For light microscopy visualization of AChR sites at MEP, peroxidase-labeled α-bungarotoxin (α-BuTX: Miami Serpentarium, Miami, FL) was prepared by the method of Nakane and Kawaoi [13]. Five mg horseradish peroxidase (grade III, Toyobo Chemical, Japan) was conjugated with 1 mg of purified α-BuTX. Peroxidase-labeled rabbit anti-human C3 (Dakopatts, Glostrup, Denmark) was used to detect C3. Fresh-frozen muscle sections (10 μm) were incubated with 100 μl of peroxidase-labeled α-BuTX at dilutions of 1:10 for 90 min at room temperature and with 100 μl of peroxidase-labeled C3 at a dilution of 1:100 for 30 min at room temperature. The treated sections were rinsed with PBS, then allowed to react with Kamovsky's diamino-benzidine (DAB) medium for 8 min. DAB reacted with the horseradish peroxidase and produced a brown precipitate. Details of this method are described in previous reports [14].

2.3. Semiquantitative analysis of the MEPs

To analyze the structural integrity of MEPs in a piece of muscle, the densities of the sites reacting with peroxidase-labeled α-BuTX, which binds quantitatively to human AChRs, were used to identify MEPs. 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 as described elsewhere [15], the mean value of the optical densities (ODs) in the straight line on MEPs (AChR "densities") and the integrated density (sum) of the OD values in the rectangle around MEPs (AChR "numbers") were derived from each endplate, after subtracting background OD values in the sarcoplasm close to the same end-plates (Fig. 1A). We assumed the "numbers" would indirectly reflect the sum of the number at AChRs. The OD values were calculated using the uncalibrated OD function of NIH imageJ2. The densities of the sites that reacted with peroxidase-labeled complement C3 (C3 "densities") were measured similarly. All measurements were performed blinded to the clinical data. The biopsied samples from nine MG patients who had definite AChR Ab detected using RIAs (RIA-AChR MG), as previously reported [16], were used as positive controls for MEP analysis.

2.4. Antibody assays

Cell-based assays (CBAs) for clustered-AChR Abs and LRP4-LUCIP assays for LRP4 Abs were performed on all available sera using previously published techniques as described in detail by Rodríguez et al. [12] and Higuchi et al. [17], respectively. Results of CBAs for clustered AChR Abs were measured on a nonlinear visual scale from 0 to 4 (0 is no signal, 0.5 is unclear, 1 is weak positive, 2 is moderate positive, 3 is strong positive, and 4 is very strong positive). These assays were performed blind to the MEP results.

2.5. Statistical analysis

Data are presented as mean ± SD. The mean differences and correlation coefficients were assessed using the Wilcoxon rank-sum test. Statistical analysis was performed using JMP Pro 13.

2.6. Data availability statement

Anonymized data can be shared after a formal review of a request from any qualified investigator.

3. Results

3.1. Patient profiles

The 20 seronegative patients (Table 1) had a female-to-male ratio of 13:7, an age of 49 ± 16 (range 17–76) years at symptom onset, and a disease duration of 20 ± 27 (range 1–84) months. Eighteen patients (90%) only had ocular symptoms, and two patients (10%) had mild limb weakness. These two patients only had a positive RNS test of the accessory nerve at 3 Hz with recording from the trapezius. The Tensilon test was positive in eight patients (40%).

3.2. Analysis of MEPs

Five (three ocular type, two generalized type) of the 20 seronegative patients had complement C3 deposited at MEPs (Fig. 1B). For AChR densities and numbers, the complement-negative and complement-positive patients were compared with nine patients with RIA-AChR MG; clinical features are shown in Table 2. As plotted in Fig. 2, the OD values for AChR densities at MEPs were 0.68 ± 0.19 in complement-positive patients, higher than those in the patients with RIA-AChR MG (0.48 ± 0.16, $n = 9$) ($P < 0.0001$) but lower than those in the complement-negative patients (1.04 ± 0.09, $n = 15$) ($P < 0.005$). Using the mean minus three SDs of the results in the complement-negative patients as the cut-off, four out of five complement-positive patients were clearly reduced. The AChR numbers, however, had wide variability

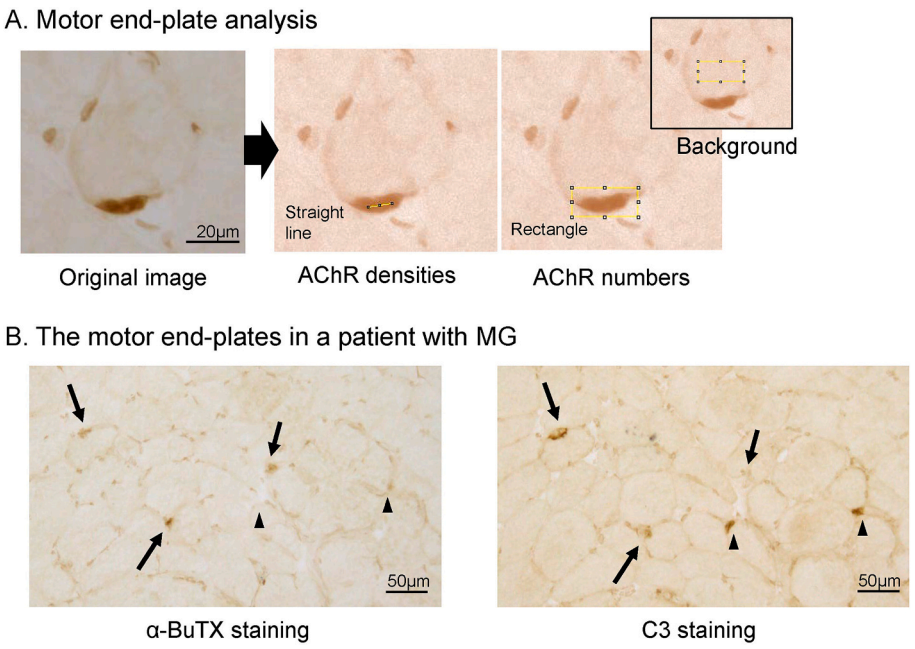

Fig. 1. Light microscopic visualization of MEPs.
(A) MEP analysis. (B) MEPs in a patient with MG: In the serial sections, AChRs labeled with peroxidase-α-BuTX are weakly stained with some deposition of complement C3 (→arrow), while AChRs are reduced in endplates with strong deposition of complement C3 (→arrowhead).

and were not clearly reduced compared to the complement-negative patients, and only three of the complement-positive patients were within the range of the patients with RIA-AChR MG (Fig. 2). There appeared to be no difference in the results between the early-onset (<50 years of age) and late-onset (>50 years of age) MG patients.

3.3. Clinical course

The five complement-positive patients had ongoing clinical symptoms and responded well to the relevant treatments. All but one of the complement-negative patients were diagnosed with other diseases (for example, cibenzoline overdosage, polymyositis, idiopathic oculomotor palsy) in follow-up over ten years (Table 1). One complement-negative patient, initially negative in all tests (no. 7 in Table 1), progressed and became seropositive for RIA-AChR Abs after 3 years, at which time re-biopsy revealed reduced AChR densities and complement deposition, and serum was positive for clustered-AChR Abs (no. 22 in Table 2).

The patient (no. 10 in Table 1) with manic-depressive psychosis had been diagnosed with MG at the other university hospital and treated with prednisone, an anticholinesterase inhibitor, and tacrolimus. As a result of the muscle biopsy, MG was ruled out, and all medications were discontinued. Since then, there was no worsening of symptoms. The patient (no. 16 in Table 1) treated as MG had no diagnosis of schizophrenia when visiting our hospital. However, two years later, he had been diagnosed with schizophrenia at another hospital. The patients (no. 9, 11, 12, 13, 14, 19, 20 in Table 1) had been treated as MG before being referred to our hospital, but these treatments were discontinued after the results of the muscle biopsy. So, 9 of 15 patients who were not diagnosed as MG had been treated as MG without success and 7 of those patients had been referred from other institutions such as the university hospital.

3.4. Antibody assays

Clustered-AChR Abs were found in two of the four patients (one patient not available) with complement deposition, and not in any of the twelve patients (three patients not available) without complement deposition (Table 1). All patients were negative by LRP4-LUCIP assays for LRP4 Abs.

4. Discussion

Here, we used a convenient quantitative measurement and applied it to biopsies from 20 seronegative patients with myasthenia-like symptoms. Five seronegative patients were positive for complement C3 deposition in MEPs, four of which had reduced AChR densities, and two (of four) had clustered-AChR Abs. The remaining 15 seronegative patients had no complement C3 deposition, reduction in AChR density, or clustered-AChR Abs. Importantly, 14 patients were confirmed to have other diseases in follow-up over ten years. These results suggest the usefulness of MEP analysis and CBA for clustered AChR Abs in diagnosing immune-mediated MG in patients with myasthenia-like symptoms, but negative routine AChR Abs.

Forty-five years ago, Engel et al. [10] reported complement deposition and reduced AChR densities at motor endplates using electron microscopy on specimens from MG patients. 20 years ago, Tsujihata et al. [16] demonstrated reduced AChR densities of AChR Ab positive MG patients by using the light microscopic method, but without examining complement deposition. Here, we found that complement deposition clearly distinguished the MG patients, with ocular or generalized MG, from those that received other diagnoses.

An unexpected but notable exception was one patient who, although negative for all tests on the first presentation, progressed and represented with typical AChR Ab-positive MG three years later. This case illustrates how careful evaluation over time may help to provide a diagnosis for some cases; a few such cases have been reported (for example, Vincent and Newsom-Davis 1985). A possible explanation, in these cases, is that the absorption of a limited number of antibodies by the patients' muscle AChRs initially results in negative serum levels. Divalent AChR Abs cause internalization of AChRs, which together with complement-activation and formation of the membrane attack complex cause loss of AChR-containing membranes and the clinical symptoms of MG [18]. Therefore, clustered-AChR CBA can be suitable for detecting antibodies that bind the AChR and that activate complement [19]. If possible seronegative MG patients should be tested by non-invasive CBAs initially.

Table 1
The clinical characteristics and examination results of 20 seronegative patients.

Patient	Age/ Sex	Symptoms	Tensilon test	RNS 3 Hz waning	C3 deposition	AChR densities	AChR numbers	Clustered-AChR-Ab scores	Final diagnosis after follow-up
1	57/F	Double vision Left ptosis	+	–	1.42	0.73	3317	0	MGFA I
2	49/M	Double vision Left ptosis	+	–	0.88	0.66	3745	No serum available	MGFA I
3	71/M	Double vision Right ptosis	+	–	1.08	0.95	1725	2.0	MGFA I
4	39/F	Bilateral ptosis Limbs weakness	+	3%	1.1	0.43	1911	0	MGFA IIIa
5	64/F	Bilateral ptosis Limbs weakness	+	4%	1.17	0.61	1261	3.5	MGFA IIIa
6	76/F	Bilateral ptosis	+	–	0	1.05	4092	No serum available	Cibenzoline overdosage
7*	27/M	Double vision Left ptosis	+	–	0	1.15	6469	0	MGFA I, positive waning on repetitive stimulation
8	60/M	Double vision Left ptosis	±	–	0	1.03	3943	No serum available	Polymyositis
9	58/F	Bilateral ptosis	+	–	0	1.15	3791	0	Thyroid-associated ophthalmopathy
10	40/F	Double vision	±	–	0	0.98	2554	0	Manic-depressive psychosis
11	62/F	Right ptosis	±	–	0	1.03	3798	No serum available	Normal biopsy, No treatment No recurrence**
12	52/F	Bilateral ptosis	±	–	0	1.10	4755	0	Normal biopsy, No treatment No recurrence**
13	46/M	Bilateral ptosis	±	–	0	1.00	3832	0	Diabetes mellitus
14	34/M	Double vision	±	–	0	1.18	8584	0	Isolated inferior rectus muscle paresis
15	45/F	Double vision Right ptosis	±	–	0	1.08	2835	0	Idiopathic oculomotor palsy
16	26/M	Bilateral ptosis	±	–	0	0.91	3301	0	Schizophrenia
17	63/F	Double vision	±	–	0	0.93	2298	0	Blepharospasm
18	60/M	Double vision	±	–	0	0.93	2700	0	Strabismus
19	17/F	Bilateral ptosis	±	–	0	0.99	2652	0	Normal biopsy, No treatment, Unknown prognosis
20	38/F	Bilateral ptosis	±	–	0	1.15	7038	0	Normal biopsy, No treatment, No recurrence**

*This patient presented with RIA-AChR MG three years later with positive complement on re-biopsy and positive clustered AChR-Abs (see no. 22 in Table 2). Two muscle biopsies were performed from the left biceps brachii. **All patients were followed-up as per availability.

Table 2
The clinical characteristics and examination results of patients with RIA-AChR MG.

Patient	Age/Sex	Tensilon test	RNS 3 Hz waning	C3 deposition	AChR densities	AChR numbers	Clustered-AChR Abs	Diagnosis
21	36/F	+	–	+	0.39	1006	Not done	MGFA I
22*	30/M	+	+	+	0.66	2494	2.0	MGFA I
23	41/F	+	–	+	0.68	2425	Not done	MGFA I
24	64/F	+	+	+	0.58	2120	Not done	MGFA I
25	26/F	+	+	+	0.44	406	Not done	MGFA IIa
26	63/F	+	+	+	0.28	740	Not done	MGFA IIb
27	46/M	+	+	+	0.23	616	Not done	MGFA IIb
28	79/F	+	+	+	0.43	1383	Not done	MGFA IIa
29	29/F	+	+	+	0.61	2046	Not done	MGFA IIa

* Patient 22 refers to Patient 7 (Table 1) after three years.

Muscular fatigue, which is a characteristic symptom of MG, is a nonspecific and subjective complaint. Myasthenia-like symptoms such as ptosis and diplopia also fluctuate within and between days, making it difficult for physicians to ascertain such symptoms objectively. Therefore, physicians can easily misdiagnose MG as having psychiatric disorders and vice versa. As our university hospital is for tertiary referral, most patients referred were challenging to diagnose, and this study included patients with psychiatric disorders as well as those without a definitive diagnosis. The patients agreed to undergo muscle biopsies despite the invasive procedure because their complaints had significantly impaired their daily lives, and we found the results helpful in providing a definite diagnosis and appropriate treatment.

As with most tests, this study has limitations. The sample size was small because only a few cases show such indetermination in the

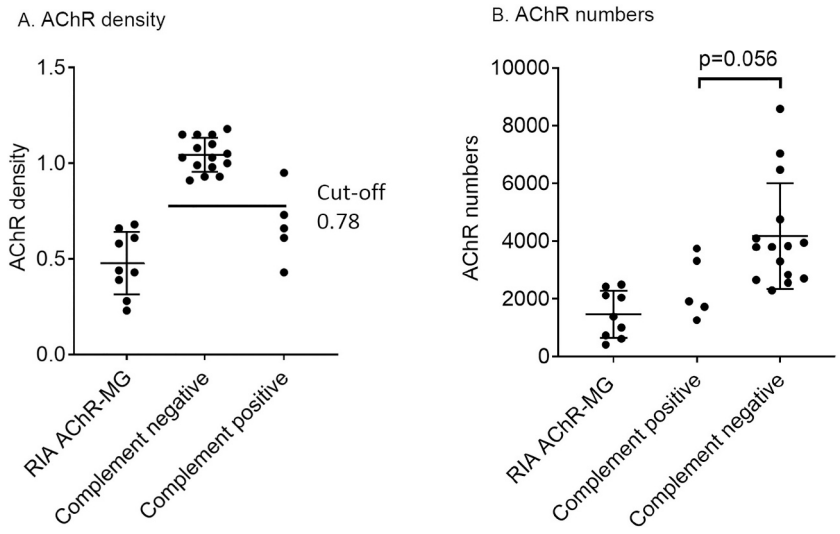

Fig. 2. The densities (A) and numbers (B) of AChRs at MEPs shown for typical RIA-AChR MG, complement-negative patients, and complement-positive patients. The cut-off for reduced densities is shown as the mean of three SDs of those in complement-negative patients. Data are presented as the mean ± SD. ODs:optical densities.

differential diagnosis of MG, and muscle biopsy is required. Then, the study was performed by only using the biceps brachii, which has relatively been easy to obtain MEPs and is not the most affected muscle. Therefore, our results might not have accurately reflected the pathology in all patients. Furthermore, a diagnosis of seronegative MG mediated by autoantibodies other than AChR Abs could not be confirmed.

5. Conclusions

MEP analysis of muscle biopsies is useful in diagnosing immune-mediated MG for seronegative patients with myasthenia-like symptoms. Ideally, clustered-AChR Ab assays, if available, should be used first, and muscle biopsies only performed when negative Ab results are found and the diagnosis remains in doubt.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

All the authors contributed to the conception and design of the study. Material preparation, data collection, and analysis were performed by Atsushi Nagaoka and Hirokazu Shiraishi. The first draft of the manuscript was written by Atsushi Nagaoka, and all the authors commented on the previous versions of the manuscript. All authors have read and approved the final manuscript.

Disclosure of potential conflicts of interest

The authors did not receive support from any organization for the submitted work.

Ethics approval

This study design was approved by the institutional review board of Nagasaki University Hospital.

Informed consent

Informed consent was not required for this retrospective study.

Consent to participate (include appropriate statements)

Informed consent was not required for this retrospective study.

Consent for publication

Informed consent was not required for this retrospective study.

Declaration of Competing Interest

None.

References

[1] A. Evoli, R. Iorio, Controversies in ocular myasthenia gravis, Front. Neurol. 11 (2020), 605902, https://doi.org/10.3389/fneur.2020.605902.
[2] M.K. Mittal, R.J. Barohn, M. Pasnoor, et al., Ocular myasthenia gravis in an academic neuro-ophthalmology clinic: clinical features and therapeutic response, J. Clin. Neuromuscul. Dis. 13 (1) (2011) 46–52, https://doi.org/10.1097/CND.0b013e31821c5634.
[3] A. Naji, M.L. Owens, Edrophonium, StatPearls, Treasure Island (FL), 2022.
[4] R.G. Whittaker, A.M. Schaefer, R.W. Taylor, et al., Differential diagnosis in ptosis and ophthalmoplegia: mitochondrial disease or myasthenia? J. Neurol. 254 (8) (2007) 1138–1139, https://doi.org/10.1007/s00415-006-0484-5.
[5] O. Aryani, M. Akbari, M. Aghsaei-Fard, et al., Oculopharyngeal muscular dystrophy misdiagnosed as myasthenia gravis: case report and review of literature, Iran J. Neurol. 16 (2) (2017) 98–99.
[6] X.F. Huang, K.Y. Wang, Z.H. Liang, et al., Clinical analysis of patients with primary Blepharospasm: a report of 100 cases in Chin, Eur. Neurol. 73 (5–6) (2015) 337–341, https://doi.org/10.1159/000381707.
[7] K. Yonemoto, S. Nomura, A. Shimizu, et al., A case of miller fisher syndrome with a false-positive edrophonium test, Rinsho Shinkeigaku 59 (6) (2019) 345–348, https://doi.org/10.5692/clinicalneurol.cn-001236.
[8] S. Suzuki, H. Murai, T. Imai, et al., Quality of life in purely ocular myasthenia in Japan, BMC Neurol. 14 (2014) 142, https://doi.org/10.1186/1471-2377-14-142.
[9] R. Ohta, M. Motomura, Autoantibodies detected in acetylcholine receptor antibody-negative myasthenia gravis, Rinsho Byori 62 (2014) 255–260.
[10] A.G. Engel, E.H. Lambert, F.M. Howard, Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations, Mayo Clin. Proc. 52 (5) (1977) 267–280.

[11] M. Tsujihata, R. Hazama, N. Ishii, et al., Limb muscle endplates in ocular myasthenia gravis: quantitative ultrastructural study, Neurology 29 (5) (1979) 654–661, https://doi.org/10.1212/wnl.29.5.654.
[12] P.M. Rodríguez Cruz, M. Al-Hajjar, S. Huda, et al., Clinical features and diagnostic usefulness of antibodies to clustered acetylcholine receptors in the diagnosis of Seronegative myasthenia gravis, JAMA Neurol. 72 (2015) 642–649, https://doi.org/10.1001/jamaneurol.2015.0203.
[13] P.K. Nakane, A. Kawaoi, Peroxidase-labeled antibody. A new method of conjugation, J. Histochem. Cytochem. 22 (12) (1974) 1084–1091, https://doi.org/10.1177/22.12.1084.
[14] M. Tsujihata, T. Yoshimura, A. Satoh, Nagataki., et al., Diagnostic significance of IgG, C3, and C9 at the limb muscle motor end-plate in minimal myasthenia gravis, Neurology 39 (10) (1989) 1359–1363, https://doi.org/10.1212/wnl.39.10.1359.
[15] A.R. Crowe, W. Yue, Semi-quantitative determination of protein expression using immunohistochemistry staining and analysis: an integrated protocol, Bio Protoc. 9 (24) (2019), e3465, https://doi.org/10.21769/BioProtoc.3465.
[16] M. Tsujihata, H. Ito, A. Satoh, et al., Semiquantitative measurement of acetylcholine receptor at the motor end-plate in myasthenia gravis, Intern. Med. 40 (2001) 376–381, https://doi.org/10.2169/internalmedicine.40.376.
[17] O. Higuchi, J. Hamuro, M. Motomura, et al., Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis, Ann. Neurol. 69 (2011) 418–422, https://doi.org/10.1002/ana.22312.
[18] W.D. Phillips, A. Vincent, Pathogenesis of myasthenia gravis: update on disease types, models, and mechanisms, F1000 Res. 5 (2016) 1513, https://doi.org/10.12688/f1000research.8206.1.
[19] M.I. Leite, S. Jacob, S. Viegas, et al., IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis, Brain 131 (Pt 7) (2008) 1940–1952, https://doi.org/10.1093/brain/awn092.