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Original Article

Cell-Based Versus Enzyme-Linked Immunosorbent Assay for the Detection of Acetylcholine Receptor Antibodies in Chinese Juvenile Myasthenia Gravis

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ABSTRACT

Background: Patients in China with juvenile-onset myasthenia gravis present early, with a high prevalence of purely ocular symptoms, spontaneous remission rates, and low antibody seropositivity. Antibody detection using a cell-based assay has been reported to increase the diagnostic sensitivity in adult-onset myasthenia gravis. However, this method in patients with juvenile-onset myasthenia gravis has not been investigated.
Methods: Patients with juvenile-onset myasthenia gravis who had not received prednisone or immunosuppressive therapy were recruited between June 2015 and April 2018 at the Huashan Hospital. Clinical information was collected. Serum anti-acetylcholine receptor antibodies were detected via cell-based assay with HEK293T cells expressing acetylcholine receptor subunits and rapsyn. Additionally, the IgG antibody subclass was identified.
Results: Eighty-two patients with juvenile-onset myasthenia gravis were enrolled in the current study. Among them, 48 patients were anti-acetylcholine receptor positive (58.5%) and 34 were seronegative (41.5%), as assessed via enzyme-linked immunosorbent assay. Cell-based assay yielded 63 positive subjects (76.8%) and 19 seronegative subjects (23.2%). All the enzyme-linked immunosorbent assay-positive samples showed robust immunofluorescence in the cell-based assay, whereas 15 of 34 enzyme-linked immunosorbent assay-negative patients (44.1%) were found to have low-affinity acetylcholine receptor antibodies. Among all the cell-based assay-positive patients, 41 were positive for both adult and fetal acetylcholine receptor antibodies (50.0%), 18 were found positive for only adult acetylcholine receptor antibodies (21.9%), and four were found to possess only fetal acetylcholine receptor antibodies (4.9%). Fifteen antibody-positive samples underwent subclassification and were confirmed to be IgG1 subclass predominant ($n = 15$, including eight adult and fetal acetylcholine receptor antibody positive, five only adult acetylcholine receptor antibody positive, and two only fetal acetylcholine receptor antibody positive). There were no significant differences in clinical features among patients with different antibody profiles.
Conclusions: The cell-based assay showed increased sensitivity in acetylcholine receptor antibody detection in Chinese patients with juvenile-onset myasthenia gravis, and most cases of Chinese juvenile-onset myasthenia gravis are still acetylcholine receptor autoantibody mediated. Furthermore, the antibodies detected are predominately of the IgG1 subclass.

Introduction

Myasthenia gravis (MG) is an autoimmune disorder that affects the function of the neuromuscular junctions, and it is mediated by autoantibodies targeting acetylcholine receptor (AChR) and other functional proteins. Fatigability and muscle weakness are clinical hallmarks.[1] MG populations can be divided into different subgroups according to several characteristics, including the age of

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onset, skeletal muscles involved, and antibody profile.[2] Juvenile-onset MG (JOMG) is characterized by an age of onset of less than 19 years, including patients with the occurrence of the first symptoms in infancy, childhood, and adolescence.[3]

JOMG shows different profiles of epidemiology, symptom, natural history, and prognosis in terms of ethnicity.[4] JOMG is much more common in East Asian than in Western populations. Epidemiological studies have indicated that JOMG accounts for 10% to 15% of MG cases in Western regions.[5] However, a much larger proportion of Chinese patients with MG present with JOMG (44.8%). The proportion of the ocular-type MG ranges from 9% to 63% in Western JOMG populations,[6-9] whereas this proportion is at least 88% in Chinese patients with JOMG.[10] In addition, Chinese patients with JOMG show lower rates of antibody seropositivity than their non-Chinese counterparts, with studies reporting 49% to 64% versus 79% to 93%, respectively.[4,10,11] In addition, it is important to note that JOMG typically shows relatively lower AChR antibody positivity when compared with adult-onset MG (AOMG).[4,12] Only 49% to 64% of JOMG cases in China were positive for AChR antibodies, whereas this figure was 85% to 90% in generalized AOMG and 50% in ocular AOMG populations.[2,10,13] JOMG cohorts from other Asian countries, Korea and Thailand, similarly show high proportion of ocular patients (97%, 93%) and benign disease course, but there is high AChR antibody positivity in Korean JOMG (AChR-binding antibodies, 89%).[14,15] Generally, Chinese patients with JOMG patients are more likely to be seronegative and experience a more benign disease course, with a higher rate of spontaneous remission.[12,16] Low AChR antibody positivity seems to be a remarkable feature of Chinese JOMG. However, whether the cause is a distinct underlying pathogenesis or the result of insensitive methodology is still unknown.

In 2008, the cell-based assay (CBA) was established[17] and showed higher sensitivity than the radioimmunoprecipitation assay and enzyme-linked immunosorbent assay (ELISA) for detecting anti-AChR.[18,19] With this advanced method, low-affinity antibodies to adult and fetal AChR could be identified in serum-negative AOMG populations of different countries and districts, ranging from 33.3% to 80%,[20-26] indicating that the so-called seronegativity is not necessarily accurate and that the use of CBA can dramatically improve sensitivity.

Thus far, the serum antibody profiles of AChR in Chinese patients with JOMG have not been investigated with CBA. Therefore the current study recruited immunotherapy-naive patients with JOMG to test for serum antibodies with the CBA method and retrospectively analyzed the clinical features according to the antibody profiles.

Materials and Methods

Patients

We enrolled patients with MG admitted to outpatient clinics in the Huashan Hospital and Fudan University, between June 2015 and April 2018. This study was approved by the ethical committee of Huashan Hospital and The Children's Hospital of Fudan University. Written informed consent was obtained from all the enrolled children and their parents.

Inclusion criteria included fluctuating weakness, age less than 15 years at the time of recruitment, and at least one of the following: (1) good response to neostigmine test or (2) greater than 10% amplitude decrement after repetitive nerve stimulation at 3 Hz.[27] Other causes were excluded in all the enrolled candidates, including congenital myasthenic syndrome, congenital myopathy, and Lambert-Eaton syndrome. Demographic information was documented, and anti-AChR titers were performed for all the patients using ELISA (EUROIMMUN, Germany). Sera from 10 patients with other neurological diseases, including stroke, neuromyelitis optica spectrum, myositis, and motor neuron disease, were collected as controls.

CBA

The pcDNA3.1-hygro vector was cloned with the cDNA of human AChR α-, β-, δ-, ε-, and γ-subunits. The p-enhanced green fluorescent protein (EGFP)-N1 vector was constructed with the cDNA of human rapsyn. All the plasmids were kindly gifted by Professor Angela Vincent and David Beeson, Nuffield Department of Clinical Neurosciences, University of Oxford. HEK293T cells were incubated in a 24-well plate, with poly-L-lysine-precoated coverslips, at 37°C for 24 hours. Plasmids containing rapsyn-EGFP and AChR α-, β-, δ-, ε-/γ- subunits were transfected in a ratio of 1:2:1:1:1/1 using lipofectamine 2000 (11668019, Invitrogen). After 48 hours of incubation, CBAs for adult and fetal AChR were prepared. Antibodies against human AChR α-(ab28489, abcam), ε- (ab65180, abcam), and γ-(Nuffield Department of Clinical Neurosciences, University of Oxford) were used to identify specific expression of adult ($\alpha2\beta\delta\varepsilon$) and fetal ($\alpha2\beta\delta\gamma$) AChR (Supplementary Figure 1).

Detection of adult and fetal AChR antibodies in serum using CBA

Sera samples were diluted at 1:20 in 1% bovine serum albumin high-glucose Dulbecco's modified Eagle's medium and seeded on the aforementioned coverslips. After one-hour incubation at 37°C, 4% formaldehyde was used to fix the cells for 15 minutes at 20°C. Anti-human IgG-Alexa Fluor 568-conjugated secondary antibody (A21090, Invitrogen) was added (1:1000) and stained for one hour at 20°C in the dark. The coverslip was placed with the cell side down on top of a drop of DAPI mounting solution (05010020, SouthernBiotech). Slides were stored in darkness at 4°C before examination and imaging via confocal (Olympus FV10) and fluorescence microscopes. Sera from patients with other neurological diseases underwent identical procedures to serve as controls. Each serum sample was tested thrice for both adult and fetal AChR-CBA. At least two consistent results out of three tests were necessary to reach a final conclusion. All seronegative samples ($n = 19$) then underwent screening for muscle-specific kinase (MuSK) and lipoprotein-related protein 4 (LRP4) antibodies using ELISA (EUROIMMUN, Germany).

Identification of IgG subclasses

As the transfected cells were cultured with serum sample and fixed by 4% formaldehyde, mouse anti-human IgG subclass antibodies were applied to the well for one hour at 20°C (anti-IgG1: I2513, Sigma-Aldrich; anti-IgG2: I 5635, Sigma-Aldrich; anti-IgG3: I7260, Sigma-Aldrich; I7385, and Sigma-Aldrich). Goat anti-mouse IgG-Alexa Fluor 568-conjugated secondary antibody (A11004, Invitrogen) was used to stain in darkness for one hour at 20°C. Following this, samples were handled in the manner previously indicated. At least two consistent results out of three tests were necessary to reach a final conclusion.

Statistical analysis

Normally distributed continuous data are presented as means and standard deviations. Differences between groups were evaluated by Kruskal-Wallis test and Fisher's exact test. P value < 0.05 was considered to be statistically significant. GraphPad Prism 5 and SPSS 17.0 were used for graphs and statistical analysis, respectively.

Results

Demographic characteristics

A total of 82 patients with JOMG were enrolled in this study, with a mean onset age of 5.8 years, ranging from one to 14 years. The mean disease duration was 1.4 months (1 to 84 months), and the female to male ratio was 1.3:1 (46:36; Table 1). The majority of patients presented purely ocular involvement ($n = 77$, 93.9%), with patients with generalized MG accounting for 6.1% of the sample (The Myasthenia Gravis Foundation of America IIa, $n = 5$). All the patients responded well to neostigmine test. In terms of repetitive nerve stimulation testing, only three candidates (3.6%) tested positive and four tested negative (4.9%). Most children were noncooperative ($n = 75$, 91.5%).

AChR profile of JOMG

Among 82 patients with JOMG, ELISA yielded 48 patients as anti-AChR positive (58.5%) and 34 as seronegative (41.5%).

CBA results revealed 63 candidates positive for AChR (76.8%) and 19 patients as seronegative (23.2%). Of these, 41 patients were positive for both adult and fetal AChR antibodies (50.0%), 18 patients were purely adult AChR antibody positive (21.9%), and four patients were purely positive for fetal AChR antibodies (4.9%; Figs 1 and 2). Besides, all the seronegative samples ($n = 19$) were also negative for MuSK and LRP4 antibodies.

When comparing ELISA and CBA results, all the ELISA-positive samples also tested positive in the CBA. However, low-affinity AChR antibodies were detected in 15 of 34 ELISA-negative patients (44.1%).

Identification of IgG subclass in seropositive patients with JOMG

Based on CBA, a total of 15 seropositive patients with JOMG were further examined by IgG subclass assay, including eight adult and fetal AChR antibody double-positive, five purely adult AChR antibody-positive, and two purely fetal AChR antibody-positive patients. All the antibodies were indicated to be of the IgG1 subclass (Fig 3).

TABLE 1.
Sample Characteristics

JOMG Patients ($N = 82$)	
Mean (S.D.) age at onset in yr	5.8 (4.24) (1-14)
Gender	
Females	46 (56.1%)
Males	36 (43.9%)
Mean (S.D.) disease duration in mo	1.4 (0.9) (1-84)
The Myasthenia Gravis Foundation of America classification	
I	77 (93.9%)
IIa	5 (6.1%)
IIb	0 (0.0%)
IIIa	0 (0.0%)
IIIb	0 (0.0%)
Iva	0 (0.0%)
IVb	0 (0.0%)
V	0 (0.0%)
RNS	
Positive	3 (3.6%)
Negative	4 (4.9%)
Noncooperative	75 (91.5%)
Neostigmine test	
Well response	82 (100.0%)
Poor response	0 (0.0%)
Thymus imaging (CT)	
Normal	80 (97.6%)
Not performed	2 (2.4%)

Abbreviations:
CT = Computed tomography
JOMG = Juvenile-onset myasthenia gravis
RNS = Repetitive nerve stimulation

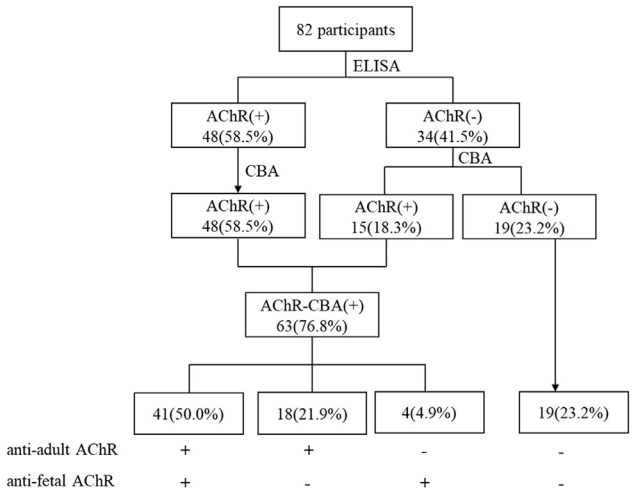

FIGURE 1. Flowchart showing profile of the AChR antibodies of the population.

Comparison of clinical characteristics among different subgroups

According to AChR antibody profiles using CBA, there were four different sero-subgroups identified: individuals positive for both adult and fetal AChR antibodies, for purely adult AChR antibody, and for purely fetal AChR antibody and seronegative. No significance of gender distribution ($P = 0.11$), age at onset ($P = 0.43$), disease duration ($P = 0.09$), or clinical manifestation ($P = 0.45$) was noted among those four subgroups (Table 2). Furthermore, according to AChR antibody profiles using both ELISA and CBA, patients could be classified into an ELISA-positive group, low-affinity group (AChR antibodies test negative through ELISA, but positive by CBA), and seronegative group. Again, no significant differences in gender distribution ($P = 0.50$), age at onset ($P = 0.99$), disease duration ($P = 0.09$), or clinical manifestation ($P = 0.50$) among subgroups were noted (Table 3).

Discussion

The diagnosis of JOMG is sometimes difficult. Except for its clinical hallmarks, a variety of examinations are required to assist diagnosis, including antibody detection, acetylcholinesterase inhibitor test, and electrophysiological testing.[4] Acetylcholinesterase inhibitor test and electrophysiological examinations work as valuable diagnostic alternatives for young children; for example, single-fiber electromyography shows abnormality in 66.7% of patients with JOMG.[27-29] However, there are very few systematic antibody studies in Chinese JOMG populations. The existing studies have reported that AChR-antibody-positive patients accounted for 49.1% and 69.5% in Chinese JOMG cohorts using ELISA.[10,30] Here CBA comes as a more sensitive assay to rescreen patients with MG who are AChR antibody negative, which further figures out more accurate antibody profiles in Chinese JOMG cohort.

Vincent et al. first reported low-affinity AChR antibodies in seronegative MG (SNMG) using CBA in 2008.[17] Since then, the antibody detection rate in patients with SNMG using CBA has ranged from 33.3% to 80%.[16,19-21,23,25] As this advanced assay uses cell lines transiently expressing clustered adult and fetal AChR, it might increase the detection sensitivity and specificity by providing

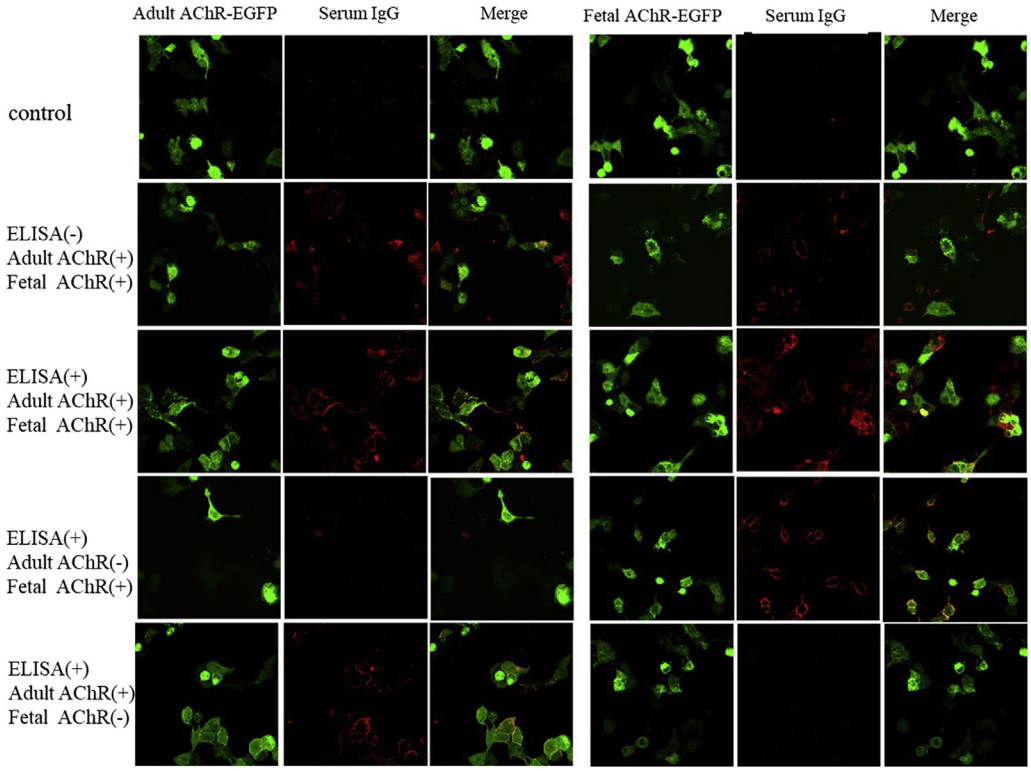

FIGURE 2. Binding of AChR IgG antibodies (red) to clustered adult- and fetal-AChR (green)-expressing HEK293T cells in patients with juvenile-onset myasthenia gravis.

native conformation at a density that imitates that at the neuromuscular junctions. *In vitro* absorption studies have further suggested that these low-affinity antibodies could be induced by unfolded subunits, rather than be conformed to AChR.[17] Thus the ability to detect the presence of these antibodies using soluble antigens at low concentration is weak. The current study found that 44.1% (15/34) of AChR-antibody-negative patients were positive using CBA. Overall, the results indicate that CBA significantly increased the seropositivity of anti-AChR antibodies in a Chinese JOMG population compared with ELISA (CBA versus ELISA: 76.8% versus 58.5%). This finding provides further evidence for the increased sensitivity of CBA when compared with ELISA, and for the fact that low-affinity AChR antibodies are a part of the pathogenesis of seronegative Chinese patients with JOMG.

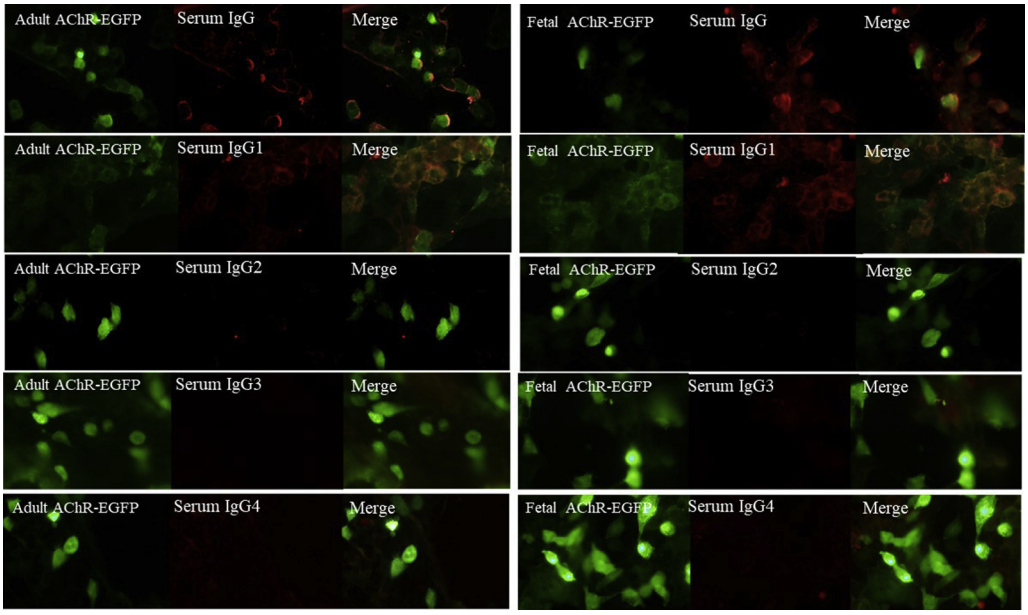

FIGURE 3. Identification of IgG subclasses. This figure takes patients with both adult and fetal AChR antibody positivity as examples. Merged images show that IgG antibodies (red) binding to adult or fetal AChR (green, EGFP fused to rapsyn) are predominantly IgG1 (yellow). EGFP, enhanced green fluorescent protein.

TABLE 2.
Comparison Among Different Subgroups

Clinical Information	Both Adult and Fetal ($n = 41$)	Purely Adult ($n = 18$)	Purely Fetal ($n = 4$)	Seronegative ($n = 19$)	*P*
Gender (F:M)	26: 15	10:8	0:4	10:9	0.11*
Age at onset (yr)	4.8 (3.3)	4.2 (2.8)	7.5 (4.4)	4.9 (3.7)	0.43†
Disease duration (mo)	1 (1-72)	2 (1-48)	9 (1-18)	1 (1-6)	0.09†
Manifestation					0.45*
OMG	39	17	3	18	
GMG	2	1	1	1	

Abbreviations:
F = Female
GMG = generalized myasthenia gravis
M = Male
OMG = ocular myasthenia gravis
* Fisher's exact test.
† Kruskal-Wallis test.

In addition, functional experiments have elucidated that the antibodies detected in patients with SNMG failed to block α-bungarotoxin binding or disrupt surface expression of AChR, but enabled activate complement,[17,31,32] suggesting that they cannot be defined as blocking or modulating antibodies. Binding of low-affinity antibodies to AChR and complement-mediated lysis might be the predominant pathological mechanisms that present different autoimmune mechanisms with ELISA-AChR-antibody-positive MG (i.e., high-affinity antibodies).[32] With respect to their clinical significance, previous research suggests that low-affinity AChR-antibody-positive MG presents distinctive clinical features from SNMG, including a higher proportion of males, earlier disease onset, and enhanced response to treatment in patients with low-affinity antibodies.[26] Our study discovered no clinical significance of gender distribution, age of onset, disease duration, or manifestation among ELISA-positive, low-affinity AChR-antibody-positive, and seronegative groups in Chinese patients with JOMG. This might either be due to the small sample size and selection bias of outpatient enrollment or be indicative of differing serological-clinical associations between JOMG and AOMG.

TABLE 3.
Comparison Among ELISA-Positive, Low-Affinity, and Seronegative Groups

Clinical Information	ELISA and CBA Positive* ($n = 48$)	CBA Positive Only† ($n = 15$)	SNMG‡ ($n = 19$)	*P*
Gender (F:M)	29: 19	7:8	10:9	0.50§
Age at onset (yr)	4.9 (3.5)	4.5 (2.6)	4.9 (3.7)	0.99‖
Disease duration (mo)	1 (1-72)	3 (1-18)	1 (1-6)	0.09‖
Manifestation				0.50§
OMG	44	15	18	
GMG	4	0	1	

Abbreviations:
ab = Antibody
AChR = Acetylcholine receptor
CBA = Cell-based assay
ELISA = Enzyme-linked immunosorbent assay
GMG = generalized myasthenia gravis
LRP4 = Lipoprotein-related protein 4
MuSK = Muscle-specific kinase
OMG = ocular myasthenia gravis
SNMG = Seronegative myasthenia gravis
* Test for AChR antibodies is positive by ELISA.
† Detection of AChR antibodies using ELISA is negative but positive by CBA.
‡ AChR-ab is negative using ELISA and CBA, together with negative MuSK and LRP4-abs.
§ Fisher's exact test.
‖ Kruskal-Wallis test.

In addition, human muscle AChR consists of five homologous subunits, with α2βγδ being the fetal form and α2βεδ being the adult AChR.[33] The CBA assay enables the examination of antibodies of specific adult and fetal forms.[21] As more than half of patients with MG had autoantibodies targeting the main immunogenic region localized on α1 subunits,[34] AChR ε and γ subunits also have been reported to be immunogenic.[35,36] For example, Beeson et al. found preferential expression of mRNA for AChR ε subunit in MG-associated thymomas, suggesting a different role of ε subunit in autoimmunization in patients with MG with thymomas.[37] In all the studies utilizing CBA, only two exhibited specific seropositivities of adult and fetal AChR antibodies.[21,38] The previous research disclosed that patients with antibodies to both adult and fetal isoforms have a greater risk of generalization.[21] We found no significant difference in clinical features, including gender, age of onset, disease duration, or clinical manifestations, among patients with different serum antibody profiles. Thus the current results do not suggest that different targets of AChR antibodies lead to different clinical phenotypes.

AChR autoantibodies are IgG1 and IgG3 subtypes.[39,40] IgG1 and IgG3 have high affinity for Fc receptors, expressed on immune cells, that have more efficiency of complement activation when compared with IgG2 and IgG4.[41] Differences between these two subclasses is the low abundance of IgG3, as it has the shortest half-life (seven days), whereas IgG1 has been proved to be the most abundant subclass in human serum.[42] In this study, low-affinity AChR antibodies in JOMG were IgG1 predominant, which is consistent with previous research.[17] Whether low-affinity AChR antibodies are purely IgG1 subclass or not needs more solid evidence to draw firm conclusions.

SNMG used to be defined as AChR-antibody-negative MG, whereas increasing studies have reported that patients with MG who are AChR antibody negative share similar pathologic changes, clinical features, and prognosis with AChR-antibody-positive MG[43-46]; limitations of the current detection assay are likely partially responsible for the lack of AChR antibodies found in patients with MG who are AChR antibody negative. This study along with other CBA-based antibody detection proves the existence of low-affinity AChR antibodies in SNMG.[17,20-22] Moreover, as MuSK antibodies and LRP4 antibodies have been, respectively, reported to be detected at 70% and ~20% in patients with MG who are AChR antibody negative,[47,48] SNMG is currently deemed to be AChR, MuSK, and LRP4 antibody triple-seronegative population.[49]

For now, the so-called triple SNMG may occur because: (1) Undetected autoantibodies, including antibodies to agrin, ColQ, cortactin, or other undiscovered antigens.[1,49] (2) False-negativity caused by insufficient CBA sensitivity.[17] To be specific, here we chose 1:20 as the serum dilution ratio, so it is certainly possible that much lower-affinity antibodies failed to be detected. (3) Involvement of non-antibody pathogenic mechanisms.[50]

The limitations of this study include its (1) noncomparative design, (2) small sizes of JOMG enrollment, (3) lack of semi-quantitative detection, and (4) subjectivity in evaluating immunofluorescence images because of lack of blindness.

In summary, this study demonstrated that Chinese patients with JOMG do not have a relatively lower antibody positivity, but possess more low-affinity antibodies, which require a more sensitive assay, such as CBA, to screen. Meanwhile, low-affinity antibodies might be related to a milder form of the condition. Therefore most Chinese cases of JOMG are still likely AChR autoantibody mediated.

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all the plasmids used in this study, including pcDNA3.1-hygro-human AChR α-, β-, δ-, ε-, and γ-subunits and p-EGFP-N1-human rapsyn, and antibodies against human AChR γ-subunits.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pediatrneurol.2019.01.016.

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