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A novel MuSK cell-based myasthenia gravis diagnostic assay

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ABSTRACT

To improve the clinical diagnosis of neural autoimmune diseases, we developed an in-house muscle-specific kinase (MuSK) antibody cell-based assay (CBA) and compared its performance with RIA, ELISA, and other CBAs. Sera from patients with myasthenia gravis (MG) and other autoimmune diseases were analyzed. We found 46 (18.3%) MuSK-CBA Ab positive cases among 251 AChR-Ab negative cases [patients] and 4 (0.6%) MuSK-CBA Ab positive cases [among] the 624 AChR-Ab positive samples. Comparing these with available clinic assays, our highly specific CBA method is more sensitive than commercial ELISA and IFA(indirect immunofluorescence assay).

1. Introduction

Myasthenia gravis (MG) is an antibody-mediated acquired autoimmune disease targeting the postsynaptic membrane at the neuromuscular junction. The degradation of postsynaptic membrane interferes with neuromuscular excitatory transmission and skeletal muscle contraction (Vincent, 2006). Approximately 80% of patients with generalized MG and 50% of patients with ocular MG have serum antibodies (Abs) against acetylcholine receptors (AChRs) (Buckley and Vincent, 2005). Antibodies against muscle-specific kinase (MuSK), another neuromuscular junction protein, were first identified in 70% of patients without the AChR antibody (Hoch et al., 2001). More recently, low-density lipoprotein receptor-related protein 4 (LRP4) and agrin have been identified as novel MG autoantigens (Li et al., 2019; Zhang et al., 2014; Zhang et al., 2012). Different types of antibodies are associated with different clinical presentations and treatment responses. In patients with MG, anti-MuSK antibodies predominantly belong to the IgG4 subclass, with features that are distinct compared with other human IgG subclasses (Zisimopoulou et al., 2013). For example, anti-MuSK IgG4 antibodies cause MG in the absence of complement activation (Cavalcante et al., 2012). Furthermore, in individual patients, MuSK-Ab titers may reflect disease activity and correlate with disease progression (Niks et al., 2008). MuSK-Ab positive MG (MuSK MG) patients frequently have severe bulbar dysfunction and respiratory insufficiency, which can be difficult to manage with immunosuppressive and immunomodulatory strategies (Mori and Shigemoto, 2013).

MuSK is a transmembrane protein associated with cytoskeleton structures on the postsynaptic membrane. It consists of four extracellular immunoglobulin (Ig) domains, an extracellular cysteine-rich domain, and an intracellular kinase domain (Masiakowski and Yancopoulos, 1998). In 2001, Werner Hoch et al. first detected MuSK-Ab in AChR-Ab negative MG serum (Hoch et al., 2001). Although radioimmunoassay (RIA) for MuSK antibodies reported in the initial study is widely adopted, its dependency on radioactive isotope limits its availability. Subsequently, a few alternate methods have been developed clinical detection of MuSK-Ab, including enzyme-linked immunosorbent assay (ELISA) (Huijbers et al., 2013), non-radioactive fluorescent immunoprecipitation assay (FIPA) (Yang et al., 2011), and cell-based assay (CBA)(Leite et al., 2008). None of these alternative assays for MuSK-Ab detection are widely available, and this restricts assessment of their respective clinic performances. Patients with triple seronegative MG (SNMG) (negative for AChR-Ab, MuSK-Ab, and LRP4-Ab based on available clinic assays) are difficult to diagnose and treat. Likely explanations for SNMG are undetected autoantibodies against undiscovered antigens or false negatives caused by insufficient detection sensitivity with current methodology. Therefore, both the identification of new potential antibodies and improvements to the sensitivity of current detection methods are important if we are to develop better

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methods for molecular diagnosis of MG.

To improve the sensitivity of current MG molecular diagnosis, we established an in-house MuSK-CBA to detect MuSK antibodies in patients with MG. Our systematic study sought to compare our in-house CBA with a commercially available MuSK-Ab radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and indirect immunofluorescence assay (IFA).

2. Materials and methods

2.1. Patients and materials

From January 2017 and December 2018, a total of 875 sera from patients with MG were analyzed at the lab of Neuroimmunology Research Institute of Medical and Pharmaceutical Sciences for MG-related antibody tests. Sera were stored at −80 °C. The diagnosis of MG involved patients presenting with criteria and subsequently, with one or both of criteria 2 and 3. These criteria are, 1) increased symptoms of muscle weakness and improved symptoms after rest; 2) in a low-frequency repetitive nerve stimulation [RNS] test, amplitude reduction > 10% or a positive neostigmine test; 3) positive AChR-Ab. The Myasthenia Gravis Foundation of America (MGFA) Classification and the MGFA post-intervention status were used to evaluate the maximum severity and treatment outcomes (Jaretzki 3rd et al., 2000). We assessed the serum for patients with a specific disease and positive results as follows: peripheral neuropathy (anti-ganglioside 1 antibodies-Ab positive, GM1-Ab positive; $n = 30$), autoimmune encephalitis [gamma-aminobutyric-acid B receptor-Ab positive ($GABA_BR$-Ab positive; $n = 5$) and anti-*N*-methyl-D-aspartate receptor-Ab positive (NMDAR-Ab positive;$n = 8$)], neuromyelitis optica spectrum disorders, NMOSD [anti-aquaporin-4-Ab positive (AQP4-Ab; $n = 23$)], idiopathic inflammatory myositis (IIM) [myositis specific autoantibody positive , MSA-Ab ($n = 15$); myositis associated autoantibody positive , MAA-Ab (n = 15)], and systemic lupus erythematosus; (SLE) [(Anti-double-stranded DNA;ds-DNA-Ab (n = 23)] and compared them with sera from healthy controls ($n = 60$).

All clinical investigations were conducted in accordance with the principles of the Helsinki Declaration, and the clinical information of all patients was kept confidential. The study was approved by the Medical Ethics Committee of Zhengzhou University. The committee waived the requirement of patient informed consent.

2.2. MuSK-CBA

See Supplementary information for details.

2.3. IFA for MuSK-Ab

We used a MuSK-Ab IFA in-vitro diagnostic kit (GA, Germany) according to the manufacturer's instructions. This is an indirect immunofluorescence test for the MuSK-Ab, in which the top and bottom rows are coated with HEp-2 MuSK-transfected (transfection rate, ~40%) and un-transfected HEp-2 cells, respectively. To rule out any nonspecific reaction, each sample was tested in parallel on the HEp-2 MuSK and untransfected HEp-2 cells. HEp-2 MuSK cells fixed onto the slide were incubated with diluted serum (1:20) from the patients' samples and the controls. Unbound components were removed with a wash step following 30 min incubation at RT. In the second step, anti-human antibodies (IgG and light chain specific) coupled with biotin were added to react specifically with bound antibodies. Excess conjugate molecules were separated from immunocomplexes bound to the fixed phase during another wash step, following 30 min incubation at RT. A third reaction step was carried out to allow the detection of the biotin coupled anti-human antibodies using streptavidin coupled to a fluorescent molecule. Excess streptavidin-fluorochrome molecules were washed following 30 min incubation at RT. The slides were covered and analyzed using confocal (Olympus FV10) and fluorescence microscopes. The cells were washed in PBS-BSA (washing solution). The fluorescence intensity was classified according to the following scheme: 3, brilliant fluorescence; 2, clear, mostly fluorescent; 1, very weak, suppressed fluorescence.

2.4. ELISA for AChR-Ab

AChR-Ab levels were measured using an AChR-Ab ELISA Kit (RSR Ltd., Cardiff, UK) following the manufacturer's instructions. A cut-off value of 0.45 nM was used to score the AChR positive sample.

2.5. ELISA and RIA for MuSK-Ab

We used the MuSK-Ab ELISA in-vitro diagnostic kit (IBL, Germany) according to the manufacturer's instructions. A cut-off value of 0.4 U/ml was used to score the MuSK positive sample. RIA for MuSK antibody concentrations were measured using the MuSK-Ab RIA Kit (RSR Ltd., Cardiff, UK) according to the manufacturer's instructions. A cut-off value of 0.05 nM was used to score MuSK positive samples.

2.6. Statistical methods

GraphPad Prism 7 (GraphPad Software, San Diego, CA) and statistics 21 (IBM Corp, Armonk, NY, USA) softwares were used to plot graphs and for statistical analyses respectively. Normally distributed continuous data were presented as means and standard deviations. Kendall's tau-b correlation coefficients were used to evaluate the relationships between the maximum severity and antibody concentrations, RIA antibody concentrations and CBA antibody titers, ELISA antibody concentrations and CBA antibody titers. $P < .05$ was considered statistically significant.

3. Results

3.1. Clinical features of patients with MG

Sera from 875 patients with MG were collected (624 AChR-Ab positive and 251 AChR-Ab negative, Fig. 1). The MuSK-CBA identified 50 MuSK-Ab positive patients: among the251 AChR-Abnegative patients, 46 (16.7%) were MuSK-CBA positive; and 4 (0.6%) MuSK-CBA positive cases discovered in 624 AChR-Ab positive MG serum. Complete clinical information from MuSK positive MG patients ($n = 23$ available from 50 positive cases)diagnosed using the new method, is shown in Table 1. The MuSK-Ab is more common in females (18/23). Further, all MuSK-Ab positive samples were from patients with generalized MG (23/23). Most MuSK-Ab positive samples were from patients with severe bulbar dysfunction (20/23) and medullary muscle involvement (13/23). In addition, 17.3% of MuSK-Ab positive samples were also AChR-Ab positive. Most were accompanied by RNS abnormalities (9/14) and positive neostigmine tests (17/19). Few MuSK-Ab positive patients(1/23) showed thymoma.

3.2. The severity of MuSK-MG and antibody concentrations

We evaluated the maximum severity of MuSK-MG based on MGFA, divided into five levels, corresponding MuSK antibody concentrations were first quantitatively tested by MuSK-ELISA. The relationship between severity of the MuSK-MG and MuSK antibody concentrations was established (Fig. 2A). Of the 23 patients with complete clinical data, among which 22 patients' sera had quantitative data of MuSK-ELISA antibody concentrations (One patient with serum MuSK antibody level below the detection limit of ELISA were excluded). Based on Kendall's tau-b (0.483; $P = .005$), the clinical severity of MuSK-MG patients is weakly associated with MuSK-ELISA antibody concentrations.

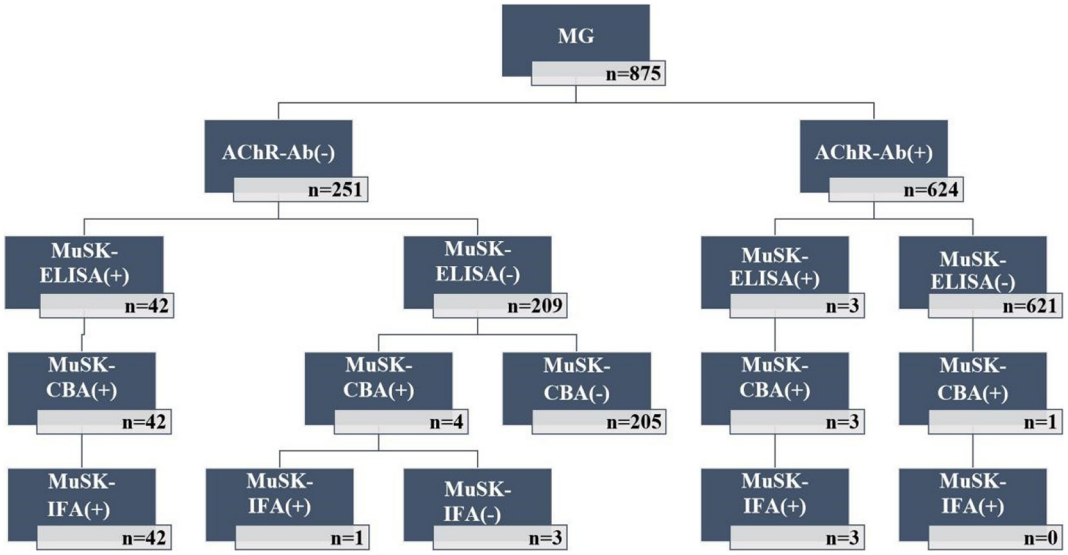

Fig. 1. Flow diagram showing detection of MuSK antibodies in MG serum by three methodologies MuSK-ELISA, in-house MuSK-CBA an IFA for the detection.

Table 1
Clinical symptoms and therapeutic responsiveness of patients with MG who were MuSK positive.

Characteristic	Males	Females	Total
	(n = 5)	(*n* = 18)	(n = 23)
Mean age in years (SD)	69.40 ± 10.01	54.17 ± 17.09	57.48 ± 16.89
Mean age at onset in years (SD)	64.60 ± 13.83	53.06 ± 17.85	55.57 ± 17.45
Mean duration of MG in years (SD)	4.82 ± 4.74	1.24 ± 2.13	2.02 ± 3.14
First symptom			
Oculobulbar	4/5	8/18	12/23
Bulbar	1/5	4/18	5/23
Limb	0/5	6/18	6/23
Clinical presentations			
Ocular	4/5	18/18	22/23
Bulbar	5/5	15/18	20/23
Limb	3/5	10/18	13/23
Generalized	3/5	9/18	12/23
Clinical subtypes			
Ocular	0/5	0/18	0/23
Generalized	5/5	18/18	23/23
MGFA status			
I	0	0	0
II	4/5	7/18	11/23
III	1/5	9/18	10/23
IV	0	1/18	1/23
V	0	1/18	1/23
AChR positive	2/5	2/18	4/23
RNS abnormalities	0/3	9/11	9/14
Thymoma present	0/5	1/18	1/23
Myasthenic crises	0/5	2/18	2/23
Neostigmine test	3/3	14/16	17/19
Treatment outcome			
CR/PR/MM	0/5	0/18	0/23
Improved	4/5	15/18	19/23
Unchanged	1/5	3/18	4/23

CR, complete remission; PR, pharmacological remission; MM, minimal manifestations.

3.3. Comparing MuSK-CBA and RIA

In order to compare the lower limits of the detection methods, four high, higher, medium, and low concentrations MuSK positive samples quantitated by ELISA and given a sufficient serum sample, were titrated. The titers of the analyzed samples were 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, and 1:10240 dilutions for both MuSK-CBA (Fig. 2B) and RIA. Interestingly, we found that when the MuSK-CBA score was higher than 1, the corresponding RIA results were all positive; while in cases where the MuSK-CBA score was < 1, the RIA results were negative.In addition,a total of 16 MG sera were tested for RIA and in-house CBA. Based on Kendall's tau-b (0.615; $P < .05$) (Fig. 2C). The RIA MuSK-Ab concentrations were weakly associated with in-house CBA dilution titers.

3.4. Comparing MuSK-CBA and ELISA

Similarly,a total of 16 MG sera were tested for MuSK-Ab concentrations with ELISA and in-house CBA. Based on Kendall's tau-b (0.957; P < .05) (Fig. 2D), the ELISA MuSK-Ab concentrationsare very strongly associated with in-house CBA dilution titers.We then tested all the 875 patients sera for detecting MuSK antibody with both in-house CBA and ELISA.Only 42 patients (16.7%) were positive for ELISA, comparing with a total of 46 (18.3%) found MuSK-CBA positive in 251 AChR-Ab negative patients ; and only 3 patients (0.48%) were positive for ELISA, comparing with a total of 4 (0.64%) found MuSK-CBA positive in 624 AChR-Ab patients (Fig. 1). MuSK-CBA has higher sensitivity since it can identify cases missed by commercial ELISA assay.

3.5. Comparing MuSK-CBA and IFA

Both MuSK-CBA and IFA methods are cell-based assays. Of the 50 MuSK-CBA positive patients who were re-tested with commercial IFA, only 46 were positive with commercial IFA (Fig. 1). The MuSK-CBA results were two-colors fluorescence based on HEK293T cells, and the results were judged based on the co-localization of green and red fluorescence (Fig. 3A). Interestingly, GFP fluorescence frequently aggregated on one side of the cells after transfection. It is ring-shaped, and the red fluorescence is also enriched on that side of the cell. The commercial IFA is based on a single-color fluorescence assay of Hep-2 cells, based on the combined green fluorescence of transfected/untransfected cells (Fig. 3B). However, we found that antinuclear antibodies in the serum of patients with systemic lupus erythematosus interfered with the results of both cell-based assays. Here, the cells are all stained with antinuclear antibodies, masking the cell surface immunofluorescence signal.

3.6. Specificity of MuSK-CBA

We assessed the sera of patients with MuSK-antibodies within the

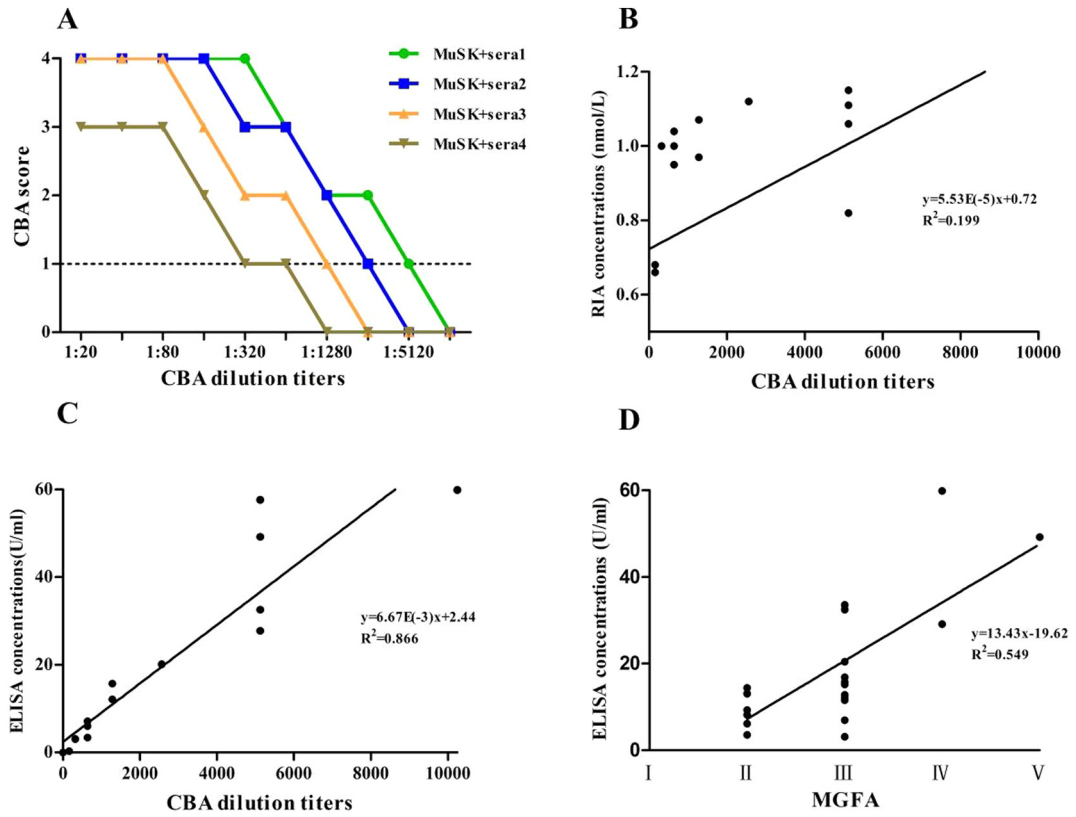

Fig. 2. Titration of MuSK positive sera (A), transfected HEK-293 cells stained with anti-MuSK sera (1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, and 1:10240 dilutions). The binding was detected with Alexa Fluor 568 anti-human IgG secondary antibody; the intensity of the fluorescence was scored (0–4). Different symbols represent serum samples from different individuals;
The RIA MuSK-Ab concentrations were weakly associated with in-house CBA dilution titers (Kendall's tau-b, $r = 0.615$; $P < .05$) (B);
The ELISA MuSK-Ab concentrations were very strongly associated with in-house CBA dilution titers (Kendall's tau-b, $r = 0.957$; $P < .05$) (C);
The MuSK-ELISA antibody concentrations and severity of the MuSK-MG (D), MGFA was divided into five levels. The x-axis 1–5 represents MGFA I-V. The severity of the MuSK-MG was weakly associated with ELISA MuSK-Ab concentrations. (Kendall's tau-b, $r = 0.483$; $P < .05$).

following four groups. Each group of diseases were accompanied by a corresponding diagnostic antibody: AChR-MG; other autoimmune neurological diseases; autoimmune neuromuscular disease; idiopathic inflammatory myositis; and systemic autoimmune disease.

For autoimmune neuromuscular disease, the MuSK-CBA can be positive with a low score. MuSK-Ab was positive only in the serum of one patient with confirmed myositis (autoantibody PL-7-positive) without diagnosed as myasthenia gravis, but not in sera from any other patients (Fig. 4).

4. Discussion

Anti-MuSK specific IgG4 concentration significantly correlated with disease severity (Bartoccioni et al., 2006; Niks et al., 2008), as demonstrated by our results which showed that 22 patients with MuSK-MG exhibited a moderate correlation with MuSK-ELISA antibody concentrations.The apparent correlation may be complicated with additional factors, such as age, gender, or concomitant diseases. Thus, improving our ability to clinically detect MuSK antibodies is very important for timely diagnosis and treatment of MG. The reported rates of patients with MG who were MuSK-Ab positive in the Chinese population vary significantly, potentially reflective of different diagnostic methodologies. For example, among patients with MG who are AChR-Ab negative, only 1 out 26 (3.8%) was identified as MuSK-Ab positive using RIA (Yeh et al., 2004). Meanwhile, a similar study identified 4 of 16 patients as MuSK-Ab positive (25%) using CBA and FIPA (Yang et al., 2011). Here, we reported the efficacy of a novel MuSK-CBA in detecting MuSK-Ab in patients with MG. MuSK-CBA had a consistently lower limit of detection, and superior sensitivity compared with ELISA (18.3% vs. 16.7%).

RIA detects the concentrations of MuSK-Ab, while CBA evaluates the MuSK-Ab dilution titers, which is a measure of the maximum dilution required for an antibody to recognize a particular epitope, and is related to the antibody affinity and avidity; RIA and ELISA are quantitative test, but have different concentrations units. ELISA results are expressed in U/mL, and the measurement of MuSK-Ab with ELISA represents the antibody titers. Therefore, the MuSK-Ab concentrations obtained by RIA was weakly associated with the dilution titers obtained by in-house CBA. However, the MuSK-Ab concentrations by ELISA associated very strongly with the in- dilution titers assessed by in-house CBA.

Pathogenic antibodies such as AChR antibody, MuSK antibody (Chang et al., 2014; Huda et al., 2017a), and LRP4 antibody (Hong et al., 2017; Li et al., 2019) can be detected with high sensitivity using RIA or similar methods. RIA is sensitive and can detect quantitatively low concentrations of MuSK antibodies, but some anti-MuSK antibodies are conformation-dependent and do not bind to the soluble MuSK extracellular domain used in this assay(Gilhus et al., 2019; Vincent et al., 2003). The high sensitivity of our CBA assay may be due to better representation of conformation-dependent epitope of MuSK. This might be maintained with high fidelity in a membrane-dependent three-dimensional structure (Ricken et al., 2018).

The MuSK-CBA has prospective clinical applications in developing countries, compared to available commercial tests. RIA has high sensitivity, but the use of radioactive isotope limits its widespread application. Commercial ELISA kits are cost-prohibitive for most hospitals in

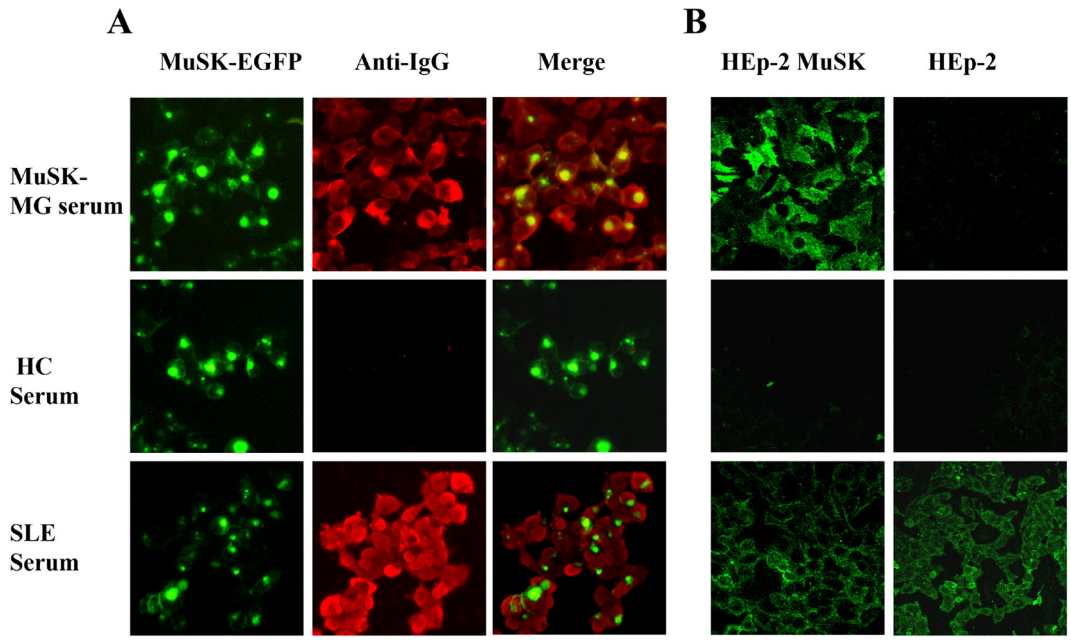

Fig. 3. Cell based assays and IFA for MuSK antibodies detection. A: Cell based assays for MuSK antibodies detection on the transfected HEK293 cell surface. HEK293 cells were transfected with MuSK-EGFP, EGFPtags exhibit a green fluorescence highlighting the transfected cells; and patient IgG binding is shown in red. A shows representative staining of anti-MuSK-Ab positive patient and patient and control serum samples, and samples exhibiting nonspecific binding from patients with systemic lupus erythematosus. B: IFA for the detection of MuSK antibodies, the two-column layout is coated with HEp-2 MuSK-transfected and un-transfected HEp-2 cells, respectively. Anti-human antibodies coupled with biotin and streptavidin coupled to a fluorescent molecule. Anti-MuSK positive serum showed a clear green fluorescence of the whole HEp-2 MuSK cell surface; conversely, the patient serum shows no fluorescence on the untransfected cells. The healthy control (anti-MuSK negative serum) samples show no or very weak fluorescence in either cell type. The presence of fluorescence in both transfected and untransfected cells against other components of HEp-2 cells was present in using serum from patients with systemic lupus erythematosus.

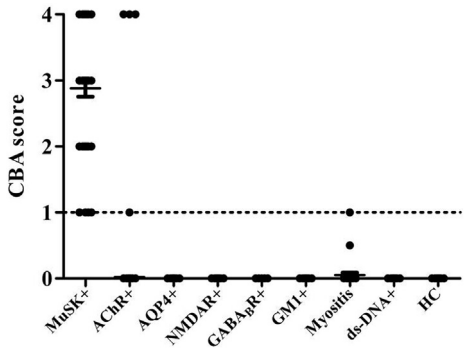

Fig. 4. MuSK-CBA scores in the cohort of serum samples. The dot plot presents the binding scores, which reflect the intensity of the fluorescence obtained using serum from MuSK-ELISA positive MG, other autoimmune diseases, and healthy controls.

developing countries, and they are hard to obtain. Although our CBA method requires cell culture, DNA transfection, and fluorescent microscopy, these technologies are available in most laboratories and are more stable and safer than radioactive compounds (Yang et al., 2011). Interestingly, we found that in-house CBA demonstrated higher sensitivity than IFA(fixed CBA). This might be caused by loss of antigenic epitopes in the commercial method or differences in fluorescence detection (Ricken et al., 2018). High sensitivity is often accompanied by low specificity. Our in-house CBA has a high specificity of 99%, the specificity here refers to the specificity of disease diagnosis rather than the methodological specificity for the comparison of the detection methods. For other neuroimmune and neuromuscular diseases, MuSK-CBA may be positive with a low score. We find one patient with confirmed idiopathic inflammatory myositis (autoantibody PL-7-positive),but without diagnosed as myasthenia gravis.This phenomenon has been reported previously (Huda et al., 2017b; Tsonis et al., 2015), suggesting an empirical cut-off value that has to be determined to satisfy both the specificity and sensitivity. Cell-based assays can be masked by the presence of antinuclear antibodies in serum or cerebrospinal fluid; the binding of a fluorescent secondary antibody to nuclear epitopes can allow for clear assessment of membrane-associated signal practically challenging. Patients with MG often exhibit other positive antibodies, such as ANA and AMA antinuclear autoantibodies (Chen et al., 1996; Omar et al., 2010; Stankovic et al., 2018), which would interfere with the results of monochromatic cell-based assays. Our new MuSK-CBA method used two-colors immunofluorescence. Green fluorescence protein was used to determine the expression of the target protein MuSK, and the co-localization of red and green fluorescence indicated a MuSK-Ab positive sample. Such co-localization could potentially be detected separately with masking nuclear or even cytoplasmic stain using quantitative morphological analysis.

Our MuSK-CBA has some limitations. CBA method demands costs in equipment investment, reagents and strong scientific quality control. Further, without strong scientific quality control, its operation is technically demanding and time-consuming, and the results may be subjective. However, use of semi-quantitative and subjective scoring techniques make it more suitable for research laboratories than for routine clinical practice. Further, this method has yet to be commercialized to allow for objective comparisons among laboratories.

5. Conclusions

Taken together, our in-house MuSK-CBA can be used as a new diagnostic assay for detecting MuSK-Ab. It is effective for detecting MuSK-Ab and more sensitive than commercial ELISA and IFA.

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Ethical standards

This study was performed in compliance with the Helsinki Declaration and its later amendments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.577076.

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