Clinical value of cell-based assays in better characterising patients with seronegative myasthenia gravis

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ABSTRACT

Objective. Patients without acetylcholine receptor (AChR) or muscle-specific kinase (MuSK) antibodies detected by radioimmunoprecipitation assays (RIA) are often classified as seronegative myasthenia gravis (SNMG). Live cell-based assays (CBAs) can detect additional antibodies to clustered AChR, MuSK and low-density lipoprotein receptor-related protein 4 (LRP4), but positivity rates are variable and both clinical relevance and utility of CBA platforms remain unclear.

Methods. Stored first sera from 82 SNMG patients were tested by live CBAs. Human embryonic kidney (HEK) cells were transfected to express clustered-AChR (adult or foetal isoforms), MuSK or LRP4, or both clustered-AChR and MuSK. The results were compared with those from 30 RIA^{+}AChR, 20 RIA^{+}MuSK patients and controls.

Results. 53/82 (72%) SNMG patients had generalised and 29/82 (28%) had ocular disease. Clustered AChR antibodies were detected in 16/82 (19.5%; including 3 patients with solely fetal AChR antibodies) and MuSK antibodies in 7/82 (8.5%) of SNMG patients. A novel, developed combined AChR-MuSK CBA efficiently detected all these antibodies. No LRP4-antibodies were identified. The detected antibodies equally distributed amongst ocular and generalised MG patients. Overall, SNMG patients with clustered-AChR antibodies, CBA-positive MuSK-MG or triple seronegative were younger and had less severe disease than seropositive patients.

Conclusion. Around one-third of SNMG patients had AChR or MuSK antibodies by live CBAs, which were efficiently detected with a convenient, combined live CBA. The results in this large and unselected cohort of MG patients demonstrate the clinical usefulness of performing CBAs and the importance of making these tests more widely available.

INTRODUCTION

Myasthenia Gravis (MG) is the most common disorder of the neuromuscular junction (NMJ), caused by autoantibodies that impair neuromuscular transmission and cause fatigable weakness of voluntary muscles. MG antibodies are directed against different proteins of the NMJ and are associated with distinct disease subtypes.[1] The standard acetylcholine receptor (AChR) radioimmunoprecipitation assay (RIA) can detect antibodies in about 80-85% of patients with generalised MG and in up to 65% with ocular MG (OMG) ,[2] with a specificity of 97–99%. The search for other antibodies in AChR-negative MG led to the identification of muscle specific kinase (MuSK) antibodies in up to 40% of AChR-negative patients, [3,4] currently detected by RIA in most laboratories. More recently, antibodies against the low-density lipoprotein receptor-related protein 4 (LRP4) have been described in a variable proportion of MG patients using different assays [5–7] and with low specificity.[8] In the last decade, the development of a high-sensitive live cell-based assay (CBA), with HEK293T cells engineered to express specific antigens, led to the detection of “clustered AChR antibodies”.[9,10] This has improved the antibody-based diagnosis of seronegative MG (SNMG), especially in paediatric and ocular cases.[11] However, to date, published studies do not quantify the extent to which live CBAs contribute to SNMG diagnosis, nor do they ask whether there are novel assay approaches to capture many antigen specificities with a single test. Previous reports have not addressed these varied aspects as they were typically based on relatively small SNMG cohorts, focused on the detection of only clustered AChR antibodies, [12–14] and only addressed either solely generalised or ocular symptoms.

Therefore, we studied a large cohort of consecutive and unselected MG patients studied over 23 years at a single tertiary centre in order to assess the presence and clinical significance of

antibodies to clustered AChR (fetal or adult form), MuSK and LRP4 by CBAs, and develop a novel CBA approach.

METHODS

Study population

Our population included 92 patients with MG seen in our clinic between 1997 and 2020, who were negative for AChR and MuSK antibodies by RIA. The clinical diagnosis of SNMG was supported by 1) evidence of neuromuscular transmission (NMT) impairment on electromyography (EMG) testing (> 10% decrement of the compound muscle action potential at repetitive nerve stimulation (RNS) or increased jitter at single fibre EMG (SF-EMG)); 2) response to cholinesterase inhibitors (ChE-I) and/or immunosuppressive treatments. Patients with symptoms and signs limited to extrinsic ocular muscles were included if brain Magnetic Resonance Imaging was unremarkable. Of the 92 patients first enrolled in the study, 10 were excluded during the follow-up based on: atypical symptoms during the disease course which excluded the diagnosis of MG (n=4); lost to follow-up in the first 2 years after disease onset (n=4); borderline positive AChR antibodies at re-testing by RIA (n=2) (Supplementary material 1). The severity of MG was evaluated at time of recruitment according to the MGFA clinical grading, [15] whilst the post-intervention status (PIS) classification[16] was used to assess the clinical state at last follow-up after the recorded treatment.

Antibody detection

The earliest serum sample available for each patient (n=92), along with 50 healthy-controls and 60 disease-controls (Neuromyelitis optica, 15; Multiple sclerosis, 10; peripheral neuropathies, 15; Amyotrophic lateral sclerosis, 13; myopathies, 7) were studied by CBAs.

Human embryonic kidney (HEK) 293T cells were transfected to express clustered-AChR (adult or fetal isoforms), MuSK or LRP4, as previously described.[9,10] In developing a combined CBA, after optimisation of co-transfection concentration of cDNA for each AChR subunit, EGFP-rapsyn and full-length EGFP-MuSK, live cells were incubated with sera from 30 RIA-positive AChR patients, 20 RIA-positive MuSK patients, 63 SNMG patients from the study cohort, 50 healthy controls, and 60 disease controls.

Statistical analysis

Statistical comparisons of demographics, clinical characteristics and antibody results were performed by Mann–Whitney test (continuous variables) or Fisher's exact test (categorical variables), as appropriate. Data distribution was assessed using Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Demographic and clinical characteristics of the SNMG population were compared with our RIA positive MuSK-MG cohort (n=103) and a sample (n=200) of our cohort of RIA positive AChR-MG (N=1104) which was extracted based on the decade of disease onset matched with the SNMG population. A P value inferior to 0.05 was considered statistically significant. Statistical analysis was performed with Graph Pad Prism v9.1 (Graph Pad Software, La Jolla, CA, USA).

RESULTS

Clinical features and antibody profile of the SNMG cohort

Eighty-two patients were included in the final analysis (Supplementary material 1). Fifty-one/ 82 (62%) patients were females, the median age of disease onset was 38.5 years (range: 6-80 years), and the median follow-up was 11.5 years (range: 2-40 years). Thymectomy had been performed in 18/82 (22%) patients before sampling, including 8/18 patients with thymic hyperplasia and one case with thymic carcinoma. Sixty-nine/82 patients (84%) responded

well to long-term ChE-I treatment and 26/82 (32%) never received immunosuppressive therapy during the follow-up.

Although only 21/82 (26%) patients were sampled at disease onset, around 50% (42/82) were untreated at sampling, reducing a possible influence of immunotherapy on these results. Antibodies against clustered AChR were found in 16/82 (19.5%), including 9 patients with antibodies only binding the adult isoform of AChR and 4 patients with antibodies only binding the fetal isoform. MuSK antibodies were detected in 7/82 (8.5%) patients, while no patient in our cohort showed positivity for LRP4 antibodies (Figures 1A-B). Thus, 59/82 (72%) SNMG patients were seronegative for clustered AChR, MuSK and LRP4 antibodies by CBA, and termed triple seronegative. Among the 29/82 (35%) patients with OMG, 10/29 (34%) were positive at CBAs, including 3 patients with MuSK and 3 with solely fetal AChR antibodies (Figure 1C). Patients' age at onset and maximum clinical severity are summarized in Figure 1D.

Clinical characteristics of the serological subgroups

The clinical features and outcome of the serological subgroups are summarised in Figure 2.

Patients with clustered AChR antibodies

The proportion of women in this subgroup was 7/16 patients (44%) and the majority of patients (13/16, 81%) were early onset, including 3 patients with paediatric onset. Thymectomy was performed in 3 patients before sampling, with evidence of thymic hyperplasia. All patients responded well to ChE-I and 7/16 (44%) never received immunosuppression. Most of the patients had a mild disease course with a good outcome. In particular, patients with antibodies directed solely to the fetal isoform of AChR mostly had

OMG with excellent clinical outcomes (** in the heatmap, Figure 2). An exception was the case of one patient with a paediatric onset who was extensively studied to exclude a congenital MG form. He refused immunotherapy and developed fixed weakness of extrinsic ocular muscles and upper limb muscles, in spite of good response to ChE-I and 3,4-diaminopyridine.

Patients with MuSK antibodies

Five/7 patients were female, with age of disease onset ranging between 17-66 years (median 39 years). All patients had mild disease course, and typical bulbar weakness at peak disease severity was found in 3/7 patients. Interestingly, 3 patients had isolated ocular disturbances characterised by bilateral ophthalmoparesis (Supplementary material 2). All patients had a good outcome, often after a short course of steroids. An exception was the case of a young woman who suffered from predominant limb weakness and didn't respond successfully to a short course of low dose steroids but had significant benefit after a trial of salbutamol.

Patients with triple seronegative MG

Fifty-nine patients were seronegative for clustered AChR, MuSK and LRP4 antibodies. Thirty-nine (65%) patients were females, with age of onset ranging between 6-72 years (median 40 years). The majority had EOMG with mild disease severity. Thymectomy was performed in 12 patients, with 5/12 (42%) of cases with thymic hyperplasia and one case of thymic carcinoma. The outcome was good in most patients, with 39/59 (66%) in minimal manifestation (MM) or better at last follow-up and 33/59 (56%) of cases who never received immunotherapy.

Comparison between serological subgroups

We compared the triple SNMG patients (n=59) with those who were positive for clustered-AChR (n=16) or MuSK (n=7) antibodies by CBAs and we found no significant difference in the demographic and clinical characteristics (Supplementary material 3).

Patients with MuSK antibodies detectable only by CBA were then compared with the RIA-positive MuSK-MG cohort (n=103) attending our centre. Patients with only CBA-positive MuSK antibodies had milder disease course than RIA-positive patients (Supplementary material 4), largely due to purely OMG cases which were absent from the RIA-positive MuSK-MG population.

In a comparison of 200 RIA-positive AChR patients (Table 1-2) with patients who had clustered AChR antibodies or triple SNMG, the latter groups were younger, more frequently ocular and with a milder disease course than RIA-positive patients, but the outcome was similar in the 2 subgroups, probably due to delayed treatment in the SNMG population.

Table 1.Comparison between clustered AChR-MG and RIA-positive AChR-MG populations

	Clustered AChR+ N=16	RIA AChR+ n=200	p
Median age at onset (IQR), years	39 (29-53)	52 (34.5-67.5)	**0.0002**
Female	7/16 (44%)	102/200 (51%)	0.746
Ocular MG	7/16 (44%)	24/200 (12%)	**0.003**
Max. MGFA			
I	7/16 (44%)	24/200 (12%)	**0.003**
II	5/16 (31%)	62/200 (32%)	1.000
III	4/16 (25%)	80/200 (40%)	0.2937
IV	0/16	9/200 (4.5%)	1.000
V	0/16	25/200 (12.5%)	0.2264
Bulbar involvement	2/16 (13%)	104/200 (52%)	**0.003**
Treatment			
No IS	7/16 (44%)	33/200 (16.5%)	**0.0141**
CS	6/16 (37%)	75/200 (37.5%)	1.000
CS+IS	3/16 (19%)	92/200 (46%)	**0.0384**
Thymectomy	3/16 (19%)	100/200 (50%)	**0.019**
Median follow-up (IQR), years	15 (6-19)	7 (3-12)	**0.0368**

PIS: MM-or-better	10/16 (63%)	139/200 (69.5%)	0.58

Table 2. Comparison between triple SNMG and RIA-positive AChR-MG populations

	tSNMG n=59	RIA AChR+ n=200	p
Median age at onset (IQR), years	39 (29-53)	52 (34.5-67.5)	**0.0002**
Female	39/59 (66%)	102/200 (51%)	**0.041**
Ocular MG	19/59 (32%)	24/200 (12%)	**0.0006**
Max. MGFA			
I	19/59 (32%)	24/200 (12%)	**0.0006**
II	23/59 (39%)	62/200 (32%)	0.2511
III	14/59 (24%)	80/200 (40%)	**0.0224**
IV	1/59 (2%)	9/200 (4.5%)	1.000
V	2/59 (3%)	25/200 (12.5%)	0.0514
Bulbar involvement	18/59 (31%)	104/200 (52%)	**0.004**
Treatment			
No IS	23/59 (39%)	33/200 (16.5%)	**0.0002**
CS	26/59 (44%)	75/200 (37.5%)	0.3634
CS+IS	10/59 (17%)	92/200 (46%)	**<0.0001**
Thymectomy	12/59 (20%)	100/200 (50%)	**<0.0001**
Median follow-up (IQR), years	12 (5-18)	7 (3-12)	**0.0012**
PIS: MM-or-better	39/59 (65%)	139/200 (69.5%)	0.621

AChR= acetylcholine receptor; CS= corticosteroids; IQR=interquartile range; MG= myasthenia gravis; MGFA=myasthenia gravis foundation of America; MM= minimal manifestation; IS=immunosuppressants; PIS= post intervention status; tSNMG= triple seronegative myasthenia gravis

AChR/MuSK combined CBA assay

First, we developed a combined assay which, simultaneously, exposes patients antibodies to both AChR and MuSK expressing HEK293T cells. By testing RIA-positive AChR and MuSK samples along with healthy controls, this showed that the combined assay retained the sensitivity and specificity of the RIA. Next, we applied the combined assay to a proportion of patients (n=63) from our SNMG cohort, finding the same sensitivity to the single assay but no further positive cases among the triple SNMG patients (figure 3A-B).

DISCUSSION

Despite advances in the detection of antibodies in MG, 10-15% of MG patients are still negative on RIA for AChR or MuSK antibodies. Here, we found that around one third of RIA-negative patients with either generalised or ocular MG were positive for clustered adult/fetal AChR or MuSK antibodies. None of the SNMG patients tested positive for LRP4 antibodies, in line with previous studies reporting a low prevalence of LRP4 antibodies in various cohorts.[17,18]

The use of CBA was successful in discriminating the antibody specificity for fetal or adult AChR, with 9 patients harbouring antibodies only against the adult isoform of AChR and 4 patients (mostly with OMG) who only had antibodies against fetal AChR. Indeed, MG sera binding preferentially to the fetal or the adult AChR subtype have been identified in other studies and may be relevant to the clinical phenotype. [19–21] Whether this is in keeping with the known expression of fetal AChRs in extraocular muscles, or whether it is because low levels of the adult AChR antibodies have been absorbed from the serum, is not known and merits further study.

The majority of patients in our cohort remained triple seronegative for clustered AChR, MuSK and LRP4 antibodies. Their clinical characteristics were heterogeneous, but these patients tended towards an earlier onset and a milder phenotype than RIA positive AChR patients, more closely resembling the clustered AChR population. However, neither triple seronegative nor clustered AChR antibody positive patients eventual showed a superior outcome to AChR-RIA positive patients. It is possible that an early serological confirmation of MG could have favoured a more aggressive therapeutic approach and a better long-term prognosis.

Based on the hypothesis that co-expression with MuSK could simultaneously detect both specificities with half the resources, without hindering expression of either, we designed a combined clustered AChR/MuSK antibody assay. As expected, this test did not increase sensitivity but it retained the same sensitivity and specificity of the individual assays. In future, we anticipate this could be used as an efficient, initial screening tool in SNMG patients. Further, given it detected all RIA-positive samples, it may be a valuable single tool to screen all MG patients.

This study has some limitations. First, its retrospective design; second, the sampling of 49% patients during immunotherapy; third, the lack of screening for congenital MG in most of our triple SNMG cases. Moreover, in comparison with RIA, CBA is currently limited to selected laboratories with specific facilities and expertise,[22] which limits its use at large scale.

The results in this large and unselected cohort of MG patients demonstrate the usefulness of performing CBAs and the importance of making these tests more widely available. Although these findings are encouraging, further studies should focus on recruiting prospective large series of SNMG immunosuppression-naive cohorts and on methodological standardization of the applied assays. At present, these assays should be reserved for SNMG patients after the exclusion of alternative diagnoses. In clinical practice, the diagnosis of MG can be challenging in patients with negative results on AChR and MuSK antibody at standard assays, highlighting the need for a consensus on clinical and electrophysiological criteria for the diagnosis of SNMG.

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CONTRIBUTORS

Study concept and design: VD, AE; acquisition of data: VD, GS, GM, MW, LJ, SF, TS, RI; AE; analysis and interpretation of data: VD, GS, GM, MW, LJ, SF, TS, PW, SRI, AV, AE drafting of the manuscript: VD, AE. critical revision of the manuscript for important intellectual content: VD, GS, PW, SRI, AV, AE; study supervision: SRI, AV, AE.

FUNDING

As per our other recent paper, Val

COMPETING INTERESTS

AV and the University of Oxford held a patent for detection of MuSK antibody assays (expired 2020), licenced to Athena Diagnostics; AV received a proportion of royalties. As per our other recent paper, Val

ETHICS APPROVAL

All patients provided consent for the use of medical data for research purposes. The study was conducted in conformation with the Helsinki Declaration and was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore. As per our other recent paper, Val

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

Figure 1. Clinical features and cell-based assay results of the SNMG cohort. CBA results scored by two observers based on the degree of cell surface fluorescence and co-localization of the red fluorescent–labelled secondary antibodies with EGFP-labelled AChRs or MuSK as previously described.[9] Given that LRP4 cDNAs did not contain EGFP, only the cell surface staining was scored (A). Clustered AChR and MuSK IgGs were detected in serum by binding (red) to the surface of live AChR-rapsyn-EGFP or MuSK-EGFP transfected HEK cells (green) (100x) (D). Serological characteristics of ocular MG patients in our cohort (C). Patients' stratification based on age of onset and maximum disease severity using the MGFA scale (D).

AChR= acetylcholine receptor; CBA= cell-based assay; EGFP= enhanced green fluorescent protein; HEK= human embryonic kidney; MG= myasthenia gravis; MGFA= myasthenia gravis foundation of America; LRP4= low-density lipoprotein receptor-related protein 4; MuSK= muscle-specific kinase; SNMG=seronegative myasthenia gravis

Figure 2. Clinical characteristics of the serological MG subgroups. Heatmap of clinical features and outcome of individual cases from our SNMG cohort stratified by antibody status and need for immunotherapy.

*=positivity for both adult and fetal AChR isoform; **=positivity only for fetal AChR; AChR= acetylcholine receptor; MGFA= myasthenia gravis foundation of America; MM= minimal manifestation; MuSK= muscle-specific kinase; PIS= post-intervention status; SNMG=seronegative myasthenia gravis

Figure 3. Single and combined AChR/MuSK assay results.

Comparison of the CBA score of the single assay versus the combined assay. The combined CBA retained the same sensitivity and specificity of the single assay (A). Fc(γ) antibody binding (red staining) of AChR-MG, MuSK-MG, and healthy control sera to AChR- EGFP-rapsyn + MuSK- EGFP (green) expressed on HEK cells. Merge of Fc(γ) IgG and AChR+MuSK-EGFP images demonstrate co-localization of MuSK-MG or AChR-MG sera but not healthy control sera (100x) (B).

AChR= acetylcholine receptor; CBA= cell-based assay; EGFP= enhanced green fluorescent protein; HEK= human embryonic kidney; IgG= immunoglobulin G; MuSK= muscle-specific kinase; RIA= radioimmunoassay; SNMG=seronegative myasthenia gravis