·Original Article·

Clinical significance of detection of antibodies to fetal and adult acetylcholine receptors in myasthenia gravis

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Abstract: Objective   To evaluate the frequency, distribution and clinical significance of the antibodies to the fetal and/or adult acetylcholine receptor (AChR) in patients with myasthenia gravis (MG). **Methods**   AChR antibodies were detected by cell-based assay in the serum of ocular MG (OMG) ($n = 90$) and generalized MG (GMG) patients ($n = 110$). The fetal-type (2α: β: γ: δ) and adult-type (2α: β: ε: δ) AChR were used as antigens, and their relevance to disease presentation was assessed. **Results**   The overall frequencies of anti-adult and anti-fetal AChR antibodies were similar in all 200 patients examined, with 14 having serum specific to the AChR-γ subunit, and 22 to the AChR-ε subunit. The overall sensitivity when using the fetal and adult AChR antibodies was higher than that when using the fetal AChR antibody only ($P = 0.015$). Compared with OMG patients, the mean age at disease onset and the positive ratio of antibodies to both isoforms of the AChR were significantly higher in patients who subsequently progressed to GMG. Older patients and patients with both anti-fetal and anti-adult AChR antibodies had a greater risk for developing generalized disease [odds ratio (OR), 1.03; 95% confidence interval (CI), 1.01–1.06 and OR, 5.09; 95% CI, 2.23–11.62]. **Conclusion**   Using both fetal- and adult-type AChRs as the antigens may be more sensitive than using either subtype. Patients with serum specific to both isoforms are at a greater risk of progressing to GMG. Patients with disease onset at an advanced age appear to have a higher frequency of GMG conversion.

Keywords: myasthenia gravis; acetylcholine receptor antibodies; acetylcholine receptor subunit; cell-based assay; adult; fetal

1   Introduction

Myasthenia gravis (MG) is an autoimmune disease mediated by antibodies to the muscle acetylcholine receptor (AChR) and other muscle antigens, which cause a neuromuscular transmission disorder[1,2]. Ptosis or double vision occurs as the initial symptom of MG in more than 75% of patients, and in nearly all patients at some time in the course of generalized MG (GMG)[3]. Up to 80% of patients who present with ocular manifestations develop generalized muscle weakness within 2 years[4-6]. However, in some patients, the symptoms are restricted to the ocular muscles for more than 2 years, and this is defined as ocular

myasthenia gravis (OMG)[7,8]. A previous study showed that the presence of anti-AChR antibody is associated with an increased risk of secondary generalization of muscular weakness[9]. However, which type of anti-AChR antibody is involved in the transition from MG with initial ocular symptom to GMG needs to be clarified.

Human muscle AChR has two subtypes, fetal and adult; anti-AChR antibodies are thus heterogeneous. Fetal AChR is composed of four subunits in a pentameric structure with 2α: β: γ: δ stoichiometry, and in the adult subtype (2α: β: ε: δ), the γ subunit is replaced by the ε subunit[10,11]. Many MG patients have a negative, equivocal, or low titer when detecting AChR antibodies using AChR from denervated human muscle that expresses mainly the fetal isoform[12]. In this study, we used a cell-based assay (CBA) to test the AChR antibodies in 90 OMG and 110 GMG patients, with fetal-type and adult-type AChRs as the antigens. The sensitivity of the assay that used both isoforms to detect anti-AChR antibodies was assessed. Some risk factors associated with secondary generalization, and their association with different types of anti-AChR antibodies and age of onset, were analyzed.

2   Materials and methods

2.1   Patients   All the MG patients included in this study were managed in the Center for Neuroinflammation, Department of Neurology, Tianjin Medical University General Hospital, China, from May 2008 to October 2011. Sera from 25 healthy Chinese blood donors and 25 patients with other neurological diseases (multiple sclerosis, 4; neuromyelitis optica, 6; clinically isolated syndrome, 4; Guillain-Barre syndrome, 6; cerebrovascular infarction, 2; and encephalitis, 3) served as controls. MG was diagnosed based on the fluctuating and fatigable weakness of voluntary muscles together with at least one of the following criteria[13]: (1) positive serum anti-AChR antibody; (2) repetitive nerve stimulation at 3 Hz showing >15% decrement of compound muscle action potentials; (3) abnormal single-fiber EMG with jitter or block; and (4) unequivocal positive response to intramuscular neostigmine. Inclusion criteria: (1) consistent with the criteria for the diagnosis

of MG; (2) disease duration of OMG patients >2 years; (3) >15 years old at serum collection; and (4) consent to participate in the study. MG patients were classified into two groups according to the clinical distribution of muscle involvement. Group I: OMG patients in whom weakness was exclusive to the eyelids and extraocular muscles and did not progress to GMG within the first 2 years. Group II: patients with GMG (disease affecting muscles besides ocular muscles)[8,14]. According to the symptoms at disease onset, patients in Group II were further divided into two subgroups. Group IIa: generalized weakness at disease onset, either subjectively or revealed during neurological examination; Group IIb: only ocular muscle involvement during the first months, but transformation to GMG later.

2.2   Materials   cDNAs encoding the human AChR subunits α, β, δ, ε and γ were cloned into pcDNA3.1/Hygro vector. Human rapsyn cDNA was cloned into pEGFP-N1. pEGFP-N1 was used as a control plasmid[14]. All the plasmids used in this study were kind gifts from Professor Angela Vincent and Professor David Beeson, Nuffield Department of Clinical Neurosciences, University of Oxford.

2.3   CBA for AChR antibodies   Detection of antibodies to the fetal-type and adult-type AChRs were conducted by CBA based on previous reports[2,15]. Human embryonic kidney 293 cells were seeded and allowed to grow for at least 24 h in 24-well plates. When the cells were 40%–50% confluent, they were transfected with fetal or adult AChR subunits, and EGFP-tagged rapsyn was used for AChR aggregation. Control cells were transfected only with pcDNA3.1/Hygro and EGFP. After 48 h, cells were incubated with patient sera diluted with DMEM (1:20) containing 20 mmol/L HEPES and 1% bovine serum albumin for 1 h at room temperature (RT). After washing three times with DMEM-HEPES, cells were fixed immediately with 3% formaldehyde for 15 min at RT. Cells were incubated with Alexa Fluor 568-conjugated goat anti-human IgG (1:750, Invitrogen-Molecular Probes, Eugene, OR) for 45 min at RT. After staining, cells were washed four times in phosphate-buffered saline, and then examined and imaged on an inverted fluorescence microscope (IX-71, Olympus,

Japan). The staining intensity and co-localization of EGFP-labeled AChR with the labeled secondary anti-human IgG (red) were evaluated independently by two investigators (QGS and ZHW). The binding to AChR was scored semi-quantitatively on a scale of 0 to 4 (see references 15 and 16 for details). The final score was the average of two independent observations (variance <1). On the basis of control results, samples scored 1 or more were classified as positive[2,15].

2.4 Statistical analysis We used GraphPad PRISM 5 for graphs and statistical analysis was performed with SPSS 17.0. Categorical variables were analyzed using the Pearson χ^2 test, while continuous variables were analyzed with Student's two-tailed *t*-test. $P < 0.05$ was considered to be statistically significant. The odds ratio of developing GMG with relevance to AChR antibodies was expressed as 95% confidence intervals. Multivariate logistic regression was used to determine whether the age at disease onset and antibodies to both fetal and adult AChR were significantly associated with the development of GMG.

3 Results

3.1 Demographic characteristics of the patients The study comprised 200 patients meeting the standard criteria for diagnosis of MG, 90 with OMG and 110 with GMG. In the OMG group (group I), there were 36 women and 54 men, and the mean age at disease onset was 43 years (range, 15–79). In group IIa, there were 35 women and 20 men, with the mean age at onset 47 years (range, 17–87). In group IIb, there were 29 women and 26 men with mean age at onset 54 years (range, 17–81). The ages at disease onset for groups I and IIb were significantly different (Table 1). In group IIb, 67.3% (37/55) of patients initially presented only with ocular muscle weakness but developed into GMG in the first year, 29.1% (16/55) transformed to GMG during the second year, and 3.6% (2/55) had generalized weakness after the first 2 years.

3.2 Determination of fetal and adult AChR antibodies Antibodies to adult or fetal AChR were all negative in the healthy subjects and disease controls. Among the 60 adult AChR antibody-negative samples, 14 were fetal AChR antibody-positive. Besides, 22 samples were positive for adult AChR antibody among 68 that failed to bind the fetal AChR antibody. In addition, 46 were negative for both adult and fetal AChR antibodies (Table 2). Among the 200 patients with MG, anti-adult antibodies were detected in 140 (70%) and anti-fetal antibodies in 132 (66%). There was no significant difference between them. The positive

Table 1. Demographic data on 200 myasthenia gravis patients

Factors	Group I	Group IIa	Group IIb	*P*
Sample size	90	55	55	
Number of male/female subjects	54/36	20/35	26/29	0.135*
Age at disease onset (years, mean ± SD)	43 ± 20	47 ± 17	54 ± 14	<0.001*

Group I, patients with ocular myasthenia gravis; Group IIa, patients with onset as generalized myasthenia gravis; Group IIb, patients with only ocular muscle involvement during the first months, but developed generalized myasthenia gravis later. *P* value between groups I and IIb.

Table 2. Determination of fetal or adult AChR-Abs in 200 patients with myasthenia gravis

	Adult AChR-Ab positive	Adult AChR-Ab negative	Total
Fetal AChR-Ab positive	118	14	132
Fetal AChR-Ab negative	22	46	68
Total	140	60	200

AChR-Ab, acetylcholine receptor antibody.

Table 3. Distribution of adult and fetal AChR antibodies in different subclinical types of myasthenia gravis patients

	Group I ($n = 90$)	Group IIa ($n = 55$)	Group IIb ($n = 55$)	P
Antibody to adult and/or fetal AChR	64 (71%)	40 (73%)	50 (91%)	0.005*
Antibody to both adult and fetal AChR	38 (42%)	35 (64%)	45 (82%)	<0.001*
Antibody to adult AChR only	14 (16%)	5 (9%)	3 (5%)	0.117*
Antibody to fetal AChR only	12 (13%)	0 (0%)	2 (4%)	0.103*

*P value between groups I and IIb.

ratio of antibodies was 77% (154/200) when both fetal and adult AChR were used as the antigens. The overall sensitivity when using adult and fetal types combined was increased by 11% compared with the result using fetal AChR antigen only ($P = 0.015$). There was a trend toward higher sensitivity in the adult and fetal AChR antigen group than in the adult AChR antigen group, but this difference did not reach significance ($P = 0.113$).

3.3 Distribution of AChR antibodies in MG patients with different clinical types The positive ratio of anti-fetal and/or anti-adult antibodies was 71% (64/90) in group I, 73% (40/55) in group IIa, and 91% (50/55) in group IIb. There was a significant difference between groups I and IIb ($P = 0.005$). The frequency of antibodies to both adult and fetal AChR was higher in group IIb (82%) than in group I (42%) ($P <0.001$) (Table 3). The risk for development of GMG increased in older patients [odds ratio (OR), 1.03; 95% confidence interval (CI), 1.01–1.06] and in patients with antibodies to both adult and fetal AChR (OR, 5.09; 95% CI, 2.23–11.62) (Table 4). Fourteen samples from group I and three from group IIb bound only with adult AChR, and five from group IIa were positive for only adult AChR. Twelve sera from group I and two from group IIb were positive for binding only to fetal AChR. No samples

Table 4. Multivariate logistic regression analysis for secondary generalization

Factor	P value	Odds ratio (95% CI)
Age at disease onset (year)	0.01	1.03 (1.01–1.06)
Antibody to both adult and fetal AChR	<0.001	5.09 (2.23–11.62)

CI, confidence interval.

in group IIa were positive for fetal AChR only. The number of patients who had antibodies binding only with adult AChR or fetal AChR did not differ significantly between groups I and IIb.

4   Discussion

In this study, we found that 37 of the 55 MG patients who initially presented only with ocular muscle weakness developed GMG within 1 year. These results are consistent with previous reports that most MG patients with initial ocular symptom progress to GMG within 1 year[5,17]. The risk for developing GMG decreased when the duration of OMG exceeded 1 year and was even lower after 2 years. To some extent, our results confirmed and also extended the previous reports. Thus, it might be more efficacious to prevent GMG during the first year.

Anti-AChR antibodies are heterogeneous. The α sub-unit is thought to be immunodominant, bearing the main immunogenic regions[18,19], and breakdown of tolerance to a self-peptide of the AChR α-subunit can induce experimental MG in rats[20]. Further support for this notion is the observation that the majority of MG serum samples bind to determinants on the α-subunit[21,22]. However, the immunogenicity of the non-α subunits has been less well studied. Since anti-AChR subunit-specific antibodies are present in MG sera[15,23], it is therefore necessary to assess the antibodies in MG patients with combined fetal and adult AChRs. Our present results not only confirmed previous reports, but also revealed the distribution of autoantibodies to the AChR ε or γ subunit in MG patients with different clinical types. In contrast to a previous report that the levator palpebrae muscles do not contain detectable AChR γ

subunits[24], some of our patients whose sera reacted only with fetal AChR did present with ptosis. It is possible that in cases with such low levels of anti-AChR antibody, some of the relevant specific antibodies had been adsorbed by the muscle end plates, so the detectable circulating antibodies were not entirely representative of those that are pathogenic. In addition, a study reported that anti-fetal AChR-specific antibodies alone are insufficient to cause ocular muscle weakness[10]. However, we found that two patients with GMG had antibodies that bound only to the AChR γ subunit. It appears that anti-fetal AChR-specific antibodies alone may be sufficient to cause generalized muscle weakness.

Conventional diagnostic radioimmunoassays use fetal AChR as the antigen[12]. Presumably, this method fails to detect antibodies that only bind to the anti-AChR ε subunit. Our results demonstrated that anti-AChR ε and anti-AChR γ subunit-specific antibodies coexist in MG patients. Compared with the fetal-type AChR antigen group, the positive ratio of AChR antibodies increased when both isoforms were used as the antigens in MG patients. Although there was no significant statistical difference, the positive ratio of AChR antibodies in the fetal and adult AChR antigen group tended to be higher than in the group with adult AChR antigen alone. This suggests that this assay, which uses both fetal and adult type AChR as the antigens, may be more sensitive than the assay that uses only fetal or adult type AChR as the antigen in MG patients.

It is known that, except for the levator palpebrae muscles, extraocular muscles express both adult and fetal isoforms of the AChR[25]. Therefore there are two kinds of anti-AChR antibodies in the serum of OMG patients, but the frequency of antibodies to both isoforms and its predictive value for OMG converting to GMG has not been well studied. We found that the positive ratios of overall AChR antibodies and those that bound to both isoforms were significantly higher in GMG patients who converted than in the OMG patients. The risk for developing GMG increased in patients with antibodies for both fetal and adult AChR, although a previous study showed that the risk of GMG was only slightly increased in patients with abnor-

mal AChR antibody levels[4]. In addition, we found that the mean age at disease onset for group IIb was significantly higher than for group I. The risk of developing GMG increased in older patients. This suggests that senior MG patients undergo conversion to GMG at a higher frequency.

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