

RESEARCH PAPER

Antibodies against the main immunogenic region of the acetylcholine receptor correlate with disease severity in myasthenia gravis

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ABSTRACT

Objective We developed an assay that detects autoantibodies against the main immunogenic region (MIR) located at the extracellular end of the nicotinic acetylcholine receptor (AChR) α subunit, and investigated its clinical relevance in myasthenia gravis (MG).

Methods In this retrospective cohort study, we measured MIR antibody (Ab) titres in sera obtained before treatment and analysed their associations with clinical parameters in 102 MG patients from two neurological centres. MIR Ab titres were determined using a modified competition immunoprecipitation assay in the presence or absence of monoclonal antibody 35.

Results 11 of 23 (47.8%) ocular type and 66 of 72 (91.7%) generalised type MG patients were positive for the presence of MIR Abs, defined as a titre $>16.8\%$ (3 SDs above the mean for 70 healthy controls). A significantly higher MIR Ab titre ($p<0.001$) was shown in generalised type ($47.9\pm 19.2\%$) rather than in ocular type MG patients ($16.4\pm 8.4\%$). Bivariate regression analysis using both titre levels of MIR Ab and routine AChR binding Ab as variables revealed MIR Abs to be an exclusive indicator positively associated with disease severity (Myasthenia Gravis Foundation of America classification, $p<0.0001$; Quantitative MG score, $p=0.008$), the presence of bulbar symptoms ($p<0.0001$) and thymoma ($p=0.016$), and negatively associated with ocular MG ($p<0.0001$).

Conclusions MIR Ab titre levels show much better correlations with factors related to disease severity compared with AChR binding Ab titres. The MIR Ab assay may be useful for predicting MG symptom severity, especially for discriminating between ocular and generalised types of MG.

INTRODUCTION

Myasthenia gravis (MG) is caused by failure of neuromuscular transmission mediated by autoantibodies (Abs) against acetylcholine receptor (AChR) and muscle-specific receptor tyrosine kinase (MuSK)¹/LDL receptor-related protein 4 (Lrp4) complex.^{2–5} The latter is a complex of AChR associated transmembrane postsynaptic proteins involved in AChR aggregation.⁴ The seropositivity rates for routine AChR binding Ab and MuSK Ab/

Lrp4 Ab in MG are 80–85% and 5–10%/<0.1% for MG patients in Japan, respectively,⁵ AChR related Abs in MG can be classified into three types: binding, blocking and modulating. The most commonly utilised and clinically useful AChR antibody assay (anti-AChR binding assay) measures IgG binding to ¹²⁵I- α -bungarotoxin labelled AChR by a radioimmunoprecipitation assay. Although the AChR binding Ab assay is useful diagnostically,⁶ titres of these Abs do not correlate well with disease severity.⁷ There appears to be some correlations between disease severity and AChR binding Ab titre on serial examinations during the clinical course of inpatient evaluation, but such correlations are variable,^{8–9} and thus binding Ab titre levels cannot predict the prognosis in individual patients. Antibodies blocking the binding site of α -bungarotoxin to AChR (blocking Abs) are detected in 50% of MG patients but blocking Ab titres correlate less well with severity, even when compared with binding Ab titres.^{10–12} On the other hand, for MuSK Ab positive MG, a correlation between MuSK Ab titre levels and disease severity was found,¹³ and the pathogenic relevance of the Abs has recently been confirmed clinically^{14–15} and experimentally.^{16–17}

The main immunogenic region (MIR) of the AChR, the principal target region of Abs, was originally defined by methods using the ability of a single rat monoclonal Ab to inhibit binding of various AChR Abs from MG patients.¹⁸ Monoclonal antibody 35 (mAb 35) is a classic example of a mAb used for detecting MIR due to its high ability to bind to the MIR, and can passively transfer experimental autoimmune MG (EAMG) in an animal model.¹⁹ The MIR targeted by mAb 35 has been shown to be located at the extracellular end of each of the two α subunits of the pentameric AChR and has been confirmed by electron microscopy.²⁰ More importantly, half or more of AChR Abs in MG patients bind to this region.²¹ Recently, MIR was shown to be a conformation dependent functional region at the extracellular end of each α subunit sequence, residues 1–81.^{22–23} Considering the potential of Abs against MIR (MIR Abs) as causative agents in MG, monitoring of these antibodies is probably useful in the clinical setting. However, the MIR Ab assay has not yet been applied clinically. Therefore, we developed

a modified method for the mAb 35 competition Ab assay²³ to detect autoantibodies against MIR (the MIR Ab assay), and investigated its clinical relevance in patients with MG.

METHODS

Patients

In this retrospective cohort study, we screened established MG patients seen at two neurological centres: Nagasaki University Hospital, from June 2008 until May 2009, and Hanamaki General Hospital, from May until August 2009, by reviewing the records of both clinical data and stored serum samples. To avoid potential bias, we enrolled consecutive patients over relatively short durations. The requirements for inclusion were as follows: frozen serum samples obtained before any treatment; complete medical records, with the Myasthenia Gravis Foundation of America (MGFA) clinical classifications at the worst conditions; continuing follow-up until study entry; and, for Hanamaki General Hospital, complete medical records with MGFA Quantitative MG score (QMG score) at serum sampling before treatment. Finally, 102 cases (Nagasaki University Hospital, n=72; Hanamaki General Hospital, n=30) met the criteria and were subjected to examinations (table 1). Recruitment of subjects at Hanamaki General Hospital was planned to analyse correlations between QMG score and serological data, excluding the influence of treatment, and to obtain clinical data blind to serological data measured at Nagasaki University. QMG score was determined by two participating neurologists (YN or KU). KU and YN are neurology specialists at Society Neurologica Japonica, and are experts in MG. At Nagasaki University Hospital, some subjects were not evaluated for QMG score before treatment and clinical data could not be analysed blind to the serological data. The worst condition for each patient was classified according to the MGFA classification and their clinical state at study entry following treatment was categorised according to MGFA post-intervention status.

The diagnosis of MG was based on clinical findings (fluctuating symptoms with easy fatigability and recovery after rest), with reduction in symptoms after intravenous administration of anticholinesterase, decremental muscle response to a train of

low frequency repetitive nerve stimuli and the presence of AChR binding Abs. The follow-up lasted 2–25 years, but there was no correlation between stored durations of serum samples and binding Ab titres (data not shown). Among 102 patients enrolled, 23 were diagnosed as having the ocular type, reconfirmation 2 years after the onset of disease.

Sera from 70 healthy controls, from disease controls including 24 MG patients with MuSK Abs and 24 Lambert–Eaton myasthenic syndrome patients with P/Q type calcium channel Abs before treatment were obtained and studied at Nagasaki University Hospital.

All clinical information and blood samples were obtained after providing informed consent, and the study protocols were approved by the ethics committees from each institution.

AChR binding Ab assay

All steps were performed at 4°C. Serum AChR binding Ab titre levels were measured by radioimmunoassay with ¹²⁵I- α -bungarotoxin using an AChR Ab kit II (Cosmic Co, Tokyo, Japan). First, 5 μ l of serum were incubated with an AChR extract labelled with 50 000 cpm of ¹²⁵I- α -bungarotoxin overnight. Excess goat antihuman immunoglobulin (IgG) serum was then added to precipitate serum antibodies. Each sample was centrifuged at 3000 rpm for 5 min and the pellets were washed three times with 20 mM phosphate buffer (pH 7.4, 0.01% TritonX-100) before counting. The radioactivity of the pellets was measured using a gamma counter (Auto Well Gamma System ARC-600; Aloca, Tokyo, Japan; 80% counting efficiency). Positive serum samples with more than 10 000 cpm precipitated were titrated at different dilutions (from 20 to 200) and re-assayed. AChR binding Ab titre levels were expressed as ¹²⁵I- α -bungarotoxin nanomoles precipitated per litre of serum with positive levels ≥ 0.5 nmol/l (3 SD above the mean for healthy controls (n=70)).

Monoclonal antibody

Monoclonal antibody 35 (mAb 35) (lot No 023K4703) was purchased from Sigma-Aldrich Inc (St Louis, Missouri, USA). Initially, we confirmed the in vivo effects of mAb 35 in animal experiments. mAb 35 (150–300 μ g) was injected intravenously into 4-week-old female Lewis rats.²⁴ Rats were examined for weight loss, muscular weakness and fatigability. Tetraplegia began to appear at 12 h, then severe weakness progressed and led to death 24–30 h after injection. Immunohistochemical studies for specimens from a motor point biopsy proved loss of AChR and deposition of complement at neuromuscular junctions.¹⁹ Using the same lot of mAb 35, we developed the MIR Ab assay.

MIR Ab assay: assay using mAb 35 competing with autoantibodies against MIR

All steps were performed at 4°C. After optimising the assay conditions, 5 μ l of patient serum were incubated with 50 000 cpm of ¹²⁵I- α -bungarotoxin labelled AChR overnight, to which 1 μ g/tube (6.67×10^{-6} mol/l) of mAb 35 was added to compete for MIR with AChR Abs for 6 h. The immune complexes of ¹²⁵I- α -bungarotoxin labelled AChR with AChR Abs and/or mAb 35 were precipitated using rabbit anti-rat IgG serum (Immuno Probe Co, Saitama, Japan), depleted with normal human sera. The precipitates were centrifuged and washed three times in 1 ml of phosphate buffer and assayed using a gamma counter. Only mAb 35 without patient serum was incubated with and measured as a zero inhibition datum. The MIR Ab titre (ie, the inhibition rate by patient serum against AChR mAb 35 binding (%)) was calculated as follows:

Table 1 Clinical characteristics of 102 myasthenia gravis patients

Characteristic	
Age (years) (mean (SD))	58.6 (17.1)
Male: female	31: 71
Age at onset (years) (mean (SD))	45.2 (20.5)
Time since onset (years) (mean (SD))	13.4 (13.1)
EOMG	43
LOMG	36
Thymoma	23
Ocular MG	23
Bulbar symptoms	45
MGFA classification (I/II/III/IV/V)	23/55/11/4/9
Current dose of PSL (mg/day) (mean (SD))	6.4 (5.7)
MGFA post-intervention status	
CSR	3
PR or better	30
MM-1 or better	40
MM-3 or better	67

CSR, complete stable remission; EOMG, early onset myasthenia gravis; LOMG, late onset myasthenia gravis; MG, myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; MM; minimal manifestations, PR; pharmacological remission; PSL, prednisolone.

$(1 - (\text{cpm of MG patient serum and mAb 35}) / (\text{cpm of only mAb 35})) \times 100$. Three SDs above the mean for healthy controls ($n=70$) was regarded as positive. The reliability of these precipitation methods was repeatedly confirmed.

Statistics

Differences between the two groups of patients were evaluated using the Student *t* test for continuous variables. Correlations were evaluated using Pearson's correlation for continuous variables or Spearman's rank correlation for categorical variables converted to numerical variables. Bivariate regression analysis using both AChR binding and MIR Abs as variables was performed to determine which was superior to the other for their relationship with clinical factors in MG patients. Values of $p < 0.05$ were considered statistically significant. All continuous data are expressed as mean \pm SD. Statistical analyses were performed using Unistat V.5.6 statistical software (Unistat, London, UK).

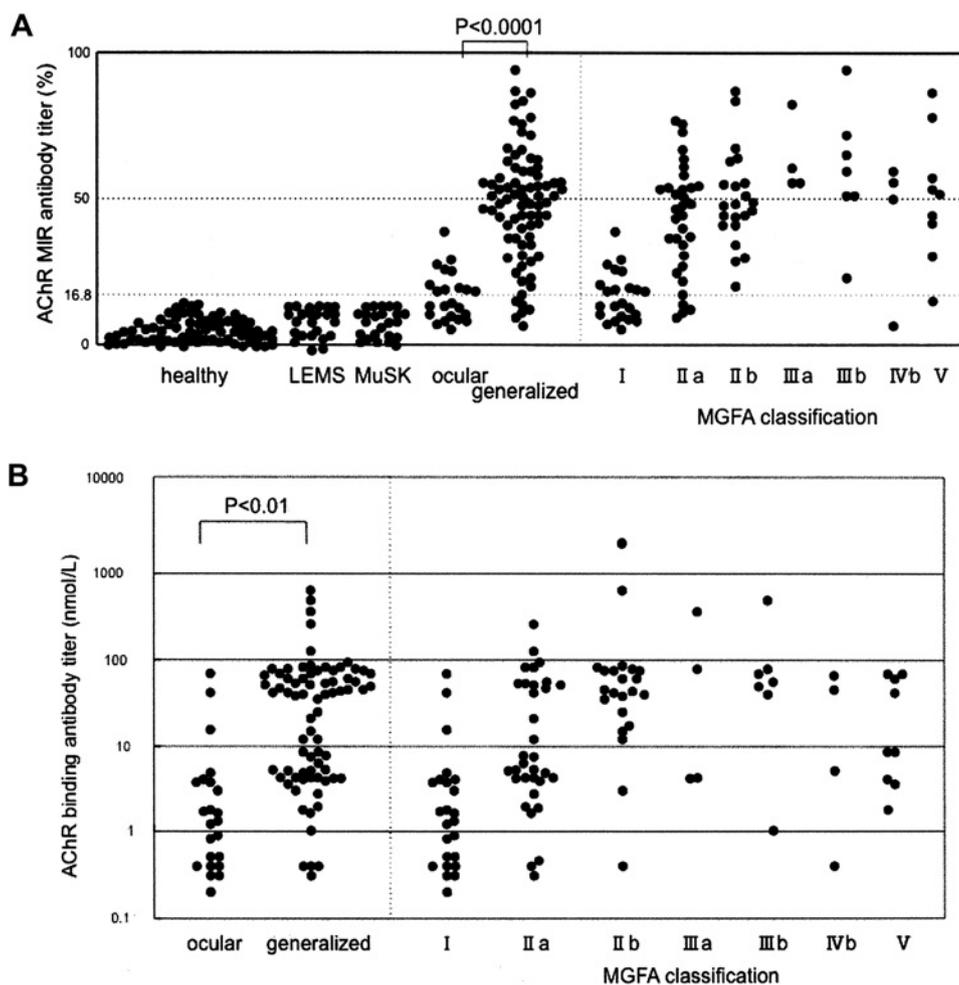
RESULTS

We had first attempted to detect MIR Ab titre (%) according to the original methods of Tzartos *et al.*²³ Their methods determine the titre as the magnitude of inhibition by mAb 35 of AChR–AChR Ab binding $(1 - (\text{cpm of MG patient serum and mAb 35}) / (\text{cpm of MG patient serum})) \times 100$, with the precipitation step using antihuman IgG serum incubated with normal rat sera, but their methods were not effective. AChR–mAb 35

complex was partly precipitated by antihuman IgG serum because reactivities against rat IgG in antihuman IgG serum could not be completely eliminated by preincubation with normal rat sera. However, when using antirat IgG serum incubated with normal human serum instead of antihuman IgG serum incubated with normal rat sera, the MIR Ab titre (%) was successfully determined in a modified way as the inhibition rate by patient serum of AChR mAb 35 binding $(1 - (\text{cpm of MG patient serum and mAb 35}) / (\text{cpm of only mAb 35})) \times 100$. After further optimising the concentration of mAb 35 and incubation time, we established the modified competition–immunoprecipitation assay using mAb 35 and stably estimated MIR Ab titre levels.

MIR Ab titre levels ranged from 0% to 94.1%. Eleven of 23 ocular type and 66 of 72 generalised type patients were positive for the presence of MIR Abs, defined as a titre $>16.8\%$ (3 SDs above the mean for healthy controls $(4.8 \pm 4.0\%)$). All disease controls with MuSK Ab positive MG ($7.2 \pm 7.2\%$, $n=24$) or P/Q type calcium channel Ab positive Lambert–Eaton myasthenic syndrome ($7.5 \pm 5.0\%$, $n=24$), were negative. The mean \pm SD of MIR Ab titre (%) in ocular and generalised MG types were $16.4 \pm 8.4\%$ and $47.9 \pm 19.2\%$, respectively (figure 1A). There was thus a significant difference in MIR Ab titre levels between ocular and generalised MG patients ($p < 0.001$, figure 1A). Furthermore, MIR Ab titre levels correlated with QMG score but AChR binding Ab titre levels did not (30 patients in a neurological centre) (tables 2 and 3). Notably, there were no ocular patients with MIR Ab titres $>40\%$. AChR binding Ab titre levels

Figure 1 (A) Main immunogenic region antibody (MIR Ab) titre levels in healthy controls ($n=70$), in Lambert–Eaton myasthenic syndrome (LEMS) patients ($n=24$), in muscle specific receptor tyrosine kinase (MuSK) positive myasthenia gravis (MG) patients ($n=24$) and in MG patients ($n=102$) as a function of Myasthenia Gravis Foundation of America (MGFA) classification. MIR Ab titre of 16.8% (mean $+3$ SD) in 70 healthy controls, broken line or greater was considered to be positive. All disease controls were negative. There was a significant difference in MIR Ab titres between ocular and generalised MG patients ($p < 0.0001$). The maximum MIR Ab titre of ocular MG was 38.1% . AChR, acetylcholine receptor. (B) Routine AChR binding Ab titre levels in MG patients as a function of MGFA classification. Binding Ab titres were overlapped in ocular and generalised patients.



Neuromuscular

Table 2 Correlations of clinical factors with main immunogenic region and binding antibody before treatment (only p values are shown for binding antibody)

Clinical factors	MIR Ab titre levels		Binding Ab titre levels p Value
	Correlation (95% CI)	p Value	
Age (years)	-0.17 (-0.36 to 0.03)	0.045*	0.28
Female sex#	0.23 (0.04 to 0.41)	0.009*	0.11
Age at onset (years)	-0.05 (-0.25 to 0.15)	0.31	0.25
Time since onset (years)	-0.15 (-0.34 to 0.05)	0.07	0.31
EOMG#	-0.01 (-0.21 to 0.19)	0.46	0.28
LOMG#	-0.17 (-0.35 to 0.03)	0.047*	0.035*
Thymoma#	0.21 (0.02 to 0.39)	0.015*	0.002*
Ocular MG#	-0.61 (-0.72 to -0.48)	<0.0001*	<0.0001*
Bulbar Symptoms#	0.44 (0.27 to 0.59)	<0.0001*	<0.0001*
QMG score (n=30)##	0.54 (0.22 to 0.75)	0.001*	0.07
MGFA classification# (at most acute presentation)	0.57 (0.43 to 0.69)	<0.0001*	<0.0001*
Crisis#	0.12 (-0.07 to 0.31)	0.10	0.34
Post-intervention status			
CSR#	-0.13 (-0.33 to 0.08)	0.10	0.14
PR or better#	-0.06 (-0.27 to 0.15)	0.28	0.15
MM-3 or better#	-0.16 (-0.36 to 0.05)	0.06	0.06

#Spearman's rank correlation.

##The number of patients having the data of QMG score is only 30 from a single study site.

*Statistically significant (p<0.05).

Pearson's correlation or #Spearman's rank correlation, n=102.

Ab, antibody; CSR, complete stable remission; EOMG, early onset myasthenia gravis; LOMG, late onset myasthenia gravis; MG, myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; MIR, main immunogenic region; MM, minimal manifestations; PR; pharmacological remission; QMG score, quantitative MG score.

were also different between ocular and generalised MG patients (p<0.01), but with considerable overlap between the two groups (figure 1B). There was a mild correlation between MIR Ab and AChR binding Ab titres (correlation coefficient=0.43, n=102) (figure 2). The levels of MIR Abs in patients who experienced myasthenic crisis (n=8) are shown as an open circle in figure 2, which shows similar levels (50.3±22.1%) to those in other patients with generalised type MG (47.6±18.9%).

Correlations of clinical factors with MIR Ab

Positive correlations with MIR Ab titre levels before treatment were found for females, the presence of thymoma, the presence of bulbar symptoms, QMG score (blinded data, n=30) and

MGFA classification at the worst condition for each patient (table 2). Negative correlations were found for age, late onset MG (≥50 years) with no thymoma and ocular type MG (table 2). Correlations of age, female sex and QMG score with binding Ab titre levels were not significant but those of other clinical factors with binding Ab (only p values are shown in table 2) showed similar results to MIR Ab. The univariate correlation analysis could not clarify the superiority of MIR or AChR binding Abs for their effects on clinical factors.

Bivariate (MIR and binding Ab) regression to clinical factors

We further performed bivariate regression analysis using both MIR and AChR binding Ab titre levels before treatment as

Table 3 Bivariate (main immunogenic region and binding antibody before treatment) regression analysis of clinical factors

Clinical factors	R Value	AChR MIR Ab		AChR binding Ab	
		Coefficient (95% CI)	p Value	Coefficient (95% CI)	p Value
Age (years)	-0.16	-0.13 (-0.30 to 0.04)	0.14	0.00 (-0.04 to 0.04)	0.91
Female sex	0.20	0.005 (0.000 to 0.009)	0.048*	-0.000 (-0.001 to 0.001)	0.52
Age at onset (years)	-0.08	-0.01 (-0.22 to 0.20)	0.91	-0.02 (-0.07 to 0.03)	0.50
Time since onset (years)	-0.19	-0.12 (-0.25 to 0.02)	0.08	0.02 (-0.01 to 0.05)	0.23
EOMG	0.11	-0.001 (-0.006 to 0.004)	0.64	0.001 (-0.001 to 0.002)	0.30
LOMG	-0.21	-0.004 (-0.009 to -0.001)	0.11	-0.000 (-0.002 to 0.001)	0.57
Thymoma	0.25	0.005 (0.001 to 0.009)	0.016*	-0.001 (-0.001 to 0.001)	0.59
Ocular MG	-0.62	-0.012 (-0.016 to -0.009)	<0.0001*	-0.000 (-0.001 to 0.001)	0.64
Bulbar symptoms	0.45	0.010 (0.006 to 0.015)	<0.0001*	0.000 (-0.001 to 0.001)	0.98
QMG score (n=30)	0.54	0.162 (0.046 to 0.278)	0.008*	-0.002 (0.030 to 0.025)	0.86
MGFA classification (at worst condition)	0.44	0.024 (0.014 to 0.035)	<0.0001*	-0.001 (-0.004 to 0.002)	0.40
Crisis	0.18	0.003 (0.000 to 0.006)	0.08	-0.000 (-0.001 to 0.000)	0.23
Post-intervention status					
CSR	-0.13	-0.001 (-0.003 to 0.001)	0.29	-0.000 (-0.001 to 0.001)	0.99
PR or better	-0.09	-0.001 (-0.007 to 0.004)	0.66	-0.000 (-0.002 to 0.001)	0.70
MM-3 or better	-0.22	-0.004 (-0.009 to 0.001)	0.08	-0.000 (-0.001 to 0.001)	0.89

*Statistically significant (p<0.05).

n=102.

Ab, antibody; AChR, acetylcholine receptor; CSR, complete stable remission; EOMG, early onset myasthenia gravis; LOMG, late onset myasthenia gravis; MG, myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; MIR, main immunogenic region; MM, minimal manifestations; PR; pharmacological remission; QMG score, quantitative MG score.

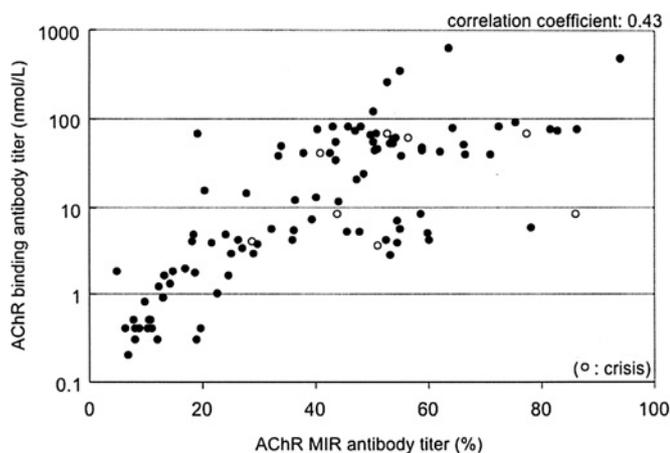


Figure 2 Correlation between main immunogenic region (MIR) and acetylcholine receptor (AChR) binding antibody titre levels (n=102).

variables to determine which Ab type was superior regarding their effects on clinical factors. This analysis clearly demonstrated that MIR Ab titre was an exclusive indicator positively associated with female sex ($p=0.048$), disease severity (QMG score and MGFA classification) ($p=0.008$, $p<0.0001$), the presence of bulbar symptoms ($p<0.0001$) and thymoma ($p=0.016$), and negatively associated with ocular MG ($p<0.0001$) (table 3).

Even if analysing only 30 patient datasets blind to the serological data, the superiority of MIR over AChR binding Abs in terms of a positive association with the presence of bulbar symptoms ($p=0.01$ vs $p=0.96$) and negative association with ocular MG ($p=0.0001$ vs $p=0.15$) remained obvious, although associations with other clinical factors did not reach significance due to the small sample size.

DISCUSSION

We developed a modified MIR Ab assay and clearly demonstrated that MIR Ab titre levels exhibit much better correlations with factors related to the severity of MG compared with AChR binding Ab titres. The original MIR Ab assay was reported by Tzartos *et al.*²³ They reported that any particular associations between MIR Ab levels and disease severity could not be found. Although there have been later reports of MIR Ab assays,^{25 26} such assays using mAbs have not yet been applied clinically. In our study, however, a significantly higher level of MIR Ab titre levels was shown in generalised type rather than in ocular type MG patients, and notably, all ocular patients showed MIR Ab levels $<40\%$. Furthermore, bivariate regression analysis revealed that higher MIR Ab levels were exclusively indicative of severe disease, with generalised symptoms and lower MIR Ab levels limited to ocular symptoms. We also found a correlation between blinded QMG score and MIR Ab but not with AChR binding Ab titre levels. These results suggest that MIR Ab levels are probably promising indicators, useful for predicting disease severity and discriminating between ocular and generalised types, and may further suggest that MIR Abs are the principal causative agents which play crucial role in the pathogenesis of MG.

It is unknown whether there exists only a single MIR on each α subunit of the AChR for pathogenic Ab binding in MG. Tzartos *et al* showed that MIR was located at the extracellular end of the α subunit, including sequence residues 66–76, and that at least half of AChR Abs in MG were directed at this MIR.²¹ On the other hand, Lennon *et al* reported the possibility

of an AChR α subunit 125–147 near the ACh binding site as a potential target for pathogenic Abs in MG patients,²⁵ but which has yet to be confirmed. In 1995, Beroukhim and Unwin reported decisive evidence supporting the localisation of MIR at the extracellular end of α subunits using electron microscopy.²⁰ They analysed them three-dimensionally and demonstrated that MIR bound with mAb 35 was localised at the extreme synaptic end of each α subunit. As this region is far from the ACh binding site,²⁷ MIR Abs do not competitively inhibit AChR function.²⁸ Recently, Luo *et al* demonstrated three-dimensional proximity in localisation between the N terminal α helix residues 1–14 and the loop residues 60–81, which suggests the conformational dependence of the antigenic structure of MIR.²⁹ Considering these results above, our MIR Ab assay probably detects Abs against the antigenic conformational structure of the extreme synaptic end of each α subunit, which crosslink to the two adjacent AChRs.

Although MG is often commented on as the best understood autoimmune disorder, the pathogenesis of ocular MG remains enigmatic.³⁰ As stated previously, at the onset of disease, approximately 50% of MG patients present with purely ocular symptoms.^{31 32} Of these patients, 50–60% finally develop generalised myasthenia; such disease courses occur within 2 years after onset in the majority of cases.^{31 32} A few studies have addressed the pathomechanisms of ocular myasthenia, analysing biopsied motor endplates^{33–35} and EAMG.^{36 37} Pathological changes in the ultrastructure of the motor endplates were observed in non-weak limb muscles of patients with ocular MG as well as in generalised MG.³⁸ There was a decrease in AChR density at the motor endplates of the biceps muscle,³⁴ and intercostal muscles,³³ even in ocular MG, although a significant difference in AChR density levels in biceps branchii muscles was observed between ocular and generalised MG.³⁵ These results suggest that the membrane destruction by complement mediated damage also occurs in ocular MG, the degree of which is weaker than that in generalised MG. Regarding the pathomechanisms of ocular myasthenia, the low titre levels of MIR Abs observed in our ocular MG patients raise two possibilities: (1) MIR Abs are actually directed against MIR in ocular MG but our assay is not sensitive enough to detect them and (2) most Abs in ocular MG are directed against unknown determinants other than the present MIR. Recently, Kaminsky's research group proposed the hypothesis that extraocular muscles could be particularly susceptible to complement mediated injury in both EAMG and MG patients.³⁹ If the present MIR is principally pathogenic and Kaminski's complement hypothesis is correct, possibility (1) appears likely.

Although limitations exist in the retrospective and partially unblinded design, the data we have presented here suggest that the MIR Ab assay is probably useful for predicting MG severity, especially for discriminating between ocular and generalised MG. Our results further suggest that the MIR is the major target region of the AChR for autoantibody responses in human MG clinically. To confirm the clinical relevance of MIR Abs and the pathological significance of MIR in MG, multicentre, prospective, double blind studies are required.

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Contributors TM designed and conducted the antibody experiments, and wrote the paper. KU and YN diagnosed the patients, conducted the statistical processing and wrote the paper. RN, MT, TF and TY diagnosed the patients and conducted the antibody experiments. MT and AK conceived the study and designed the experiments. MM conceived the study, designed the experiments and wrote the paper.

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Competing interests None.

Ethics approval The ethics committees from Nagasaki University and Hanamaki General Hospital approved the study.

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