Autoantibody to dihydropyridine receptor in myasthenia gravis

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Abstract

To investigate autoantibodies related to excitation–contraction (E-C) coupling in patients with myasthenia gravis (MG), we developed a novel method to detect autoantibodies against dihydropyridine receptor (DHPR). Using this method, we detected DHPR antibody in 37% (11 out of 30) of MG patients with thymoma. Antibodies were not detected in normal nor disease controls. The titer of DHPR antibodies showed no significant correlation with autoantibodies to acetylcholine nor ryanodine receptors. The DHPR antibody is another marker for thymoma in MG, and it might have some role in clinical symptoms related to E-C coupling.

Key words; Myasthenia gravis, dihydropyridine receptor, ryanodine receptor, thymoma, autoantibody, exciation-contraction coupling

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease with impaired neuromuscular transmission mainly due to the effect of autoantibodies to acetylcholine receptor (AChR) (Conti-Fine et al., 2006). Recently, dysregulation of sarcoplasmic reticulum (SR) Ca^{2+} relaease has been associated with muscle fatigue (Bellinger et al., 2008). An electrophysiological study showed that fatigue in MG patients was caused not only by abnormal neuromuscular transmission but also by impairment of excitation-contraction (E-C) coupling (Pagala et al., 1993). In the process of E-C coupling in skeletal muscle, ryanodine receptor (RyR) and dihydropyridine receptor (DHPR) function as Ca^{2+} channels (Takeshima et al., 1994). Autoantibody against RyR (RyR Ab) was previously detected from sera of MG patients with a large majority of them being associated with thymoma (Mygland et al., 1992; Takamori et al., 2004). However, RyR is intracellular protein, and evidence that RyR Abs are pathogenic *in vivo* is still missing (Skeie et al., 2006). Therefore, a new method to detect autoantibodies against other E-C coupling-related proteins facing to outside of muscle cells is necessary to explain the pathogenesis of the E-C coupling-impairment in MG. In this study, we developed a new method to detect specific autoantibodies against DHPR (DHPR Abs) and studied their frequency in MG patients.

2. Patients and Methods

2.1. Patients

We examined serum samples from 57 patients with MG (male: 22; female: 35, with thymoma: 30; without thymoma: 27, ocular: 13; generalized: 44, AChR Ab-positive: 48; AChR Ab-negative: 9 [1; muscle-specific kinase (MuSK) Ab-positive], mean of onset age; 51.9 year-old), 14 patients with Lambert-Eaton myasthenic syndrome (LEMS), 35 disease controls (thymoma without MG: 7, other neurological disorders: 28) and 20 age-matched normal subjects. The clinical histories and laboratory data of patients were fully collected and reviewed by our physicians for the accuracy of diagnosis. Serum samples were drawn before starts of any immunosuppressive therapies and stocked at $-20°C$ until measurement. Their titers of AChR Ab were measured by immunoprecipitation assay using ^{125}I-α-bungarotoxin labeled AChR from TE671 (Lindstrom et al., 1976). Their titers of anti-P/Q-type voltage-gated calcium channel (VGCC) antibody (P/Q-VGCC Ab) were measured by immunoprecipitation assay using ^{125}I-ω-conotoxin MVIIC labeled P/Q-type VGCC from rat cerebellum (Motomura et al., 1997).

2.2 DHPR Ab and RyR Ab

We used the ELISA starter accessory package (Bethyl Laboratories,

Montgomery, TX) to perform sandwich ELISA procedure. The monoclonal antibody (mAbs) to DHPR (clone 1A, Sigma Aldrich Inc., St Louis, MI) (Morton and Froehner, 1987) (Morton et al., 1988) and horseradish peroxidase (HRP)-conjugated anti-human immunoglobuline G (IgG) antibody (ICN Pharmaceuticals Inc., Aurora, OH) were utilized. A crude extract of DHPRs / RyRs was obtained from the skeletal muscle of rabbits as previously described (Inui et al., 1987; Iwasa, 1997; Saito et al., 1984). We found DHPR in R2 fraction and RyR in R3 fraction by the western blotting method with mAbs to each protein (clones 1A and 34C respectively). A microtiter plate was coated with mAbs to DHPR (2 µg/ml) or negative control included BSA for 1 hour and was blocked with the postcoat solution buffer (Bethyl Laboratories) for 30 minutes. The plate was then incubated with the DHPR extract (R2 fraction). After these steps, human sera were applied to the plates. Then, we performed standard procedure for ELISA. The optical density (OD)-values were read at 450 nm with Multiskan MS-UV (Labsystems, Helsinki, Finland). The result is expressed as net OD in which the value of negative control, coated with BSA, was subtracted. Titers that exceeded the average + 3X standard deviation of normal samples were defined as the positive. To optimize assay procedure, we examined titers with various volumes of applied samples from a DHPR Ab-positive patient and a normal subject. We examined what OD was obtained on the ELISA using commercial anti-DHPR polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). We also tested the DHPR Ab-positive and -negative samples using a control monoclonal antibody (anti-KLH mAb, clone KLH-60, Sigma Aldrich Japan, Tokyo, Japan) before the DHPR extract is added. We also

detected DHPR Abs qualitatively by the western blotting method with standard procedure using the DHPR extract (R2 fraction) as the antigen and sample sera or mAbs (clone 1A) to DHPRs as the antibodies. Their titers of RyR Abs were measured by sandwich ELISA assay procedure same as for DHPR Abs, using mAbs to RyRs (clone 34C, Sigma-Aldrich Inc., Saint Louis, MI) and the RyR extract (R3 fraction).

2.3. Statistics

Antibody-positive rates were analyzed using Fisher's exact probability test. The correlation of the titer of DHPR Abs and other kinds of antibodies was elucidated using Pearson product-moment correlation test.

3. Result

Of 30 patients of MG with thymoma (T-MG), 11 patients (37%) were positive for DHPR Abs (Figure 1).   On the other hand all samples from MG without thymoma (N-MG), disease control (DC) and normal control (NC) were negative.   The titers of positive sera changed depending on the volume of applied sample (Figure 2A).   The titers of commercial anti-DHPR polyclonal antibodies changed depending on the volume of applied sample (Figure 2B).   The anti-DHPR Ab in samples was not detectable if anti-KLH mAb was used as coating antibody instead of anti-DHPR mAb (Figure 2C).   The western blotting method also detected DHPR Abs in samples that were DHPR Ab-positive by this procedure (Figure 2D).

Even though we did not detect of DHPR Abs from samples without AChR Abs, there was no significant correlation between these two autoantibodies (Figure 3A).   There was no significant correlation between DHPR Abs and RyR Abs in all samples.   However, if only the DHPR Ab-positive samples are taken, there was significant correlation between DHPR Abs and RyR Abs (Figure 3B).   Additionally, P/Q-VGCC Abs were not detected in samples with DHPR Abs.

In T-MG, the positive rate for DHPR Ab was higher in female than male (Table).   Of 11 patients of DHPR Abs-positive, only one patient was ocular type and others were generalized type.   However, no difference in positive rates was found in patients with late-onset (after 40 years of age) and in those with early-onset.   Moreover, there was no difference of MG onset age or MG ADL score between DHPR Ab-positive

and -negative patients with T-MG (Figure 4A, 4B). Concerning to the WHO classification of thymoma, of 11 DHPR Ab-positive patients, three had B1, four had B2, three had B3 and one had unknown thymoma (Figure 4C).

4. Discussion

In this study, we developed a new measurement method to detect autoantibodies against DHPR. Using this method, we could detect DHPR Abs from 37% of T-MG samples. All tested samples were drown before any immunological therapies. In T-MG, the positive rate of this antibody has tendencies to be high in female and in generalized type. All samples with DHPR Abs have also AChR and RyR Abs. However, the titers of these antibodies do not have significant correlation with each other. Moreover, the sequence of DHPR has no significant similarity with those of AChR (Noda et al., 1983) or RyR (Tanabe et al., 1987; Zorzato et al., 1990). On the other hand, there was no sample that was positive for both DHPR Ab and P/Q-VGCC Abs, in spite of several significant similar regions between these two types of VGCC (Perez-Reyes et al., 1992; Tanabe et al., 1987)

So far, DHPR Abs have not been measured in MG sera. However, there were several methods for the detection of autoantibodies to AChRs from sera with MG (Lindstrom et al., 1976). The immunoprecipitation assay could detect antibodies to the complex of AChR and α-bungarotoxin, which is a specific ligand to AChR. After that, the ELISA method using anti-AChR mAbs was designed without utilization of the neurotoxin and radio-isotope (Dwyer et al., 1983). Using this ELISA method, with some modification, we could detect disease specific Abs from T-MG sera in this study, with a specificity of 100% and a sensitivity of 37%. Since the mAbs (clone 1A) bound to DHPRs specifically (Morton et al., 1988), the Abs we detected in patients sera was

thought to have specificity to DHPR. There was no correlation between the titers of DHPR Abs and other antibodies detected from MG or LEMS sera in our study. These results suggest that DHPR Abs could be produced independently in the patients with T-MG. However, if only the DHPR Ab-positive patients are taken, there is correlation with RyR Ab titers ($p<0.05$). There might be two different populations in T-MG patients with RyR Ab.

In general, physiological processes which lead to muscle contraction, include electrical (nerve conduction, neuromuscular transmission and muscle membrane excitation) and mechanical (E-C coupling and muscle contractility) responses (Pagala et al., 1993). Fatigue in MG patients is caused by impairment of E-C coupling as well as abnormal neuromuscular transmission (Pagala et al., 1993). Both RyR and DHPR are calcium channels participated in E-C coupling of skeletal muscle (Takeshima et al., 1994). From some sera of patients with MG, RyR Abs could be detectable (Takamori et al., 2004). However, since RyR is intracellular protein, the evidence that RyR Abs are pathogenic *in vivo* is still missing (Skeie et al., 2006). On the other hand, since DHPR is a membrane protein facing to outside of cytoplasm,(Grabner et al., 1998) DHPR Abs that we noted here could easily access targets and be pathogenic for the impairment of E-C coupling. Our method is useful to detect antibodies against DHPRs and would contribute to clarification of the roles of antibodies in another pathogenesis of MG.

Further accumulation of patients and physiological evaluation of E-C coupling of these patients should be addressed.

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Figure legend

Figure 1.

Titers of DHPR Ab.

Titers that exceeded the average + 3 x standard deviation of normal samples (broken bar) were defined as the positive. Eleven out of 30 patients (37%) with T-MG were positive for DHPR Ab. On the other hand, all of N-MG, LEMS, DC and NC were negative.

Figure 2.

Detection of DHPR Ab

A. Titers with various volumes of applied samples. OD values of MG sample with DHPR Ab increased depending on the applied volume. On the other hand, OD values of NC sample did not increase in spite of the change of the applied volume.

B. Titers with various concentration of commercial anti-DHPR polyclonal IgG. OD values of DHPR IgG increased depending on the applied volume.

C. OD value on the control assay. The DHPR Ab positive samples did not show significant titers (over the value of mean + 3 x SD of control samples) on the assay using anti-KLH mAb.

D. DHPR Ab detected with the western blotting method

Western blotting method also could detect DHPR Ab from DHPR Ab positive samples by our method [lane 2] and did not detect DHPR Ab from NC samples [lane 3]. Lane

1; anti-DHPR monoclonal antibody (arrow head), Lane 2; sample of DHPR Ab positive by our method, Lane 3; NC sample.

DC; disease control, DHPR; dihydropyridine receptor, DHPR Ab; autoantibody to DHPR, KLH; keyhole limpet hemocyanin, LEMS; Lambert-Eaton myasthenic syndrome, MG; myasthenia gravis, NC; normal control, N-MG; MG with normal thymus, SD; standard deviation, T-MG; MG with thymoma

Figure 3.

Correlation between titers of DHPR Ab and other autoantibodies.

A. DHPR Ab and AChR Ab. There was no correlation between titers of DHPR-Ab and AChR-Ab (n=35).

B. DHPR Ab and RyR Ab. There was not so significant correlation between titers of DHPR Ab and RyR Ab (n=57). However, if only the DHPR Ab-positive patients are taken, there is correlation with RyR Ab titers (bar; the linear regression, n=11, r=0.61, p<0.05).

AChR Ab; autoantibody to acetylcholine receptor, DHPR Ab; autoantibody to dihydropyridine receptor, DHPR Ab (+); DHPR Ab positive, DHPR Ab (-); DHPR Ab negative, MG; myasthenia gravis, RyR Ab; autoantibody to ryanodine receptor.

Figure 4.

DHPR Ab and clinical features of MG patients.

A. Onset age of MG in patients with and without DHPR Ab. There was no difference of

MG onset ages between DHPR Ab-positive and -negative patients with T-MG.

B. MG ADL score in patients with and without DHPR Ab.　There is no difference of

MG ADL score between DHPR Ab-positive and -negative patients with T-MG.

C. DHPR Ab and thymoma types.　Of 11 DHPR Ab-positive patients, three had B1,

four had B2, three had B3 and one had unknown types by the WHO classification of

thymoma.

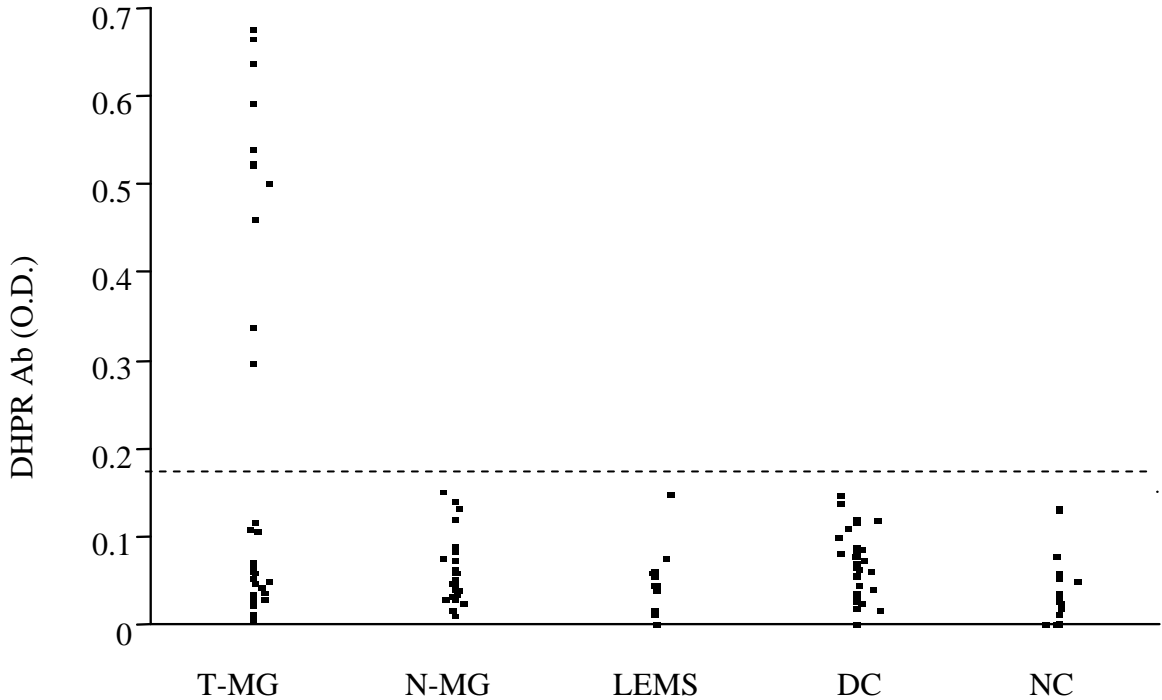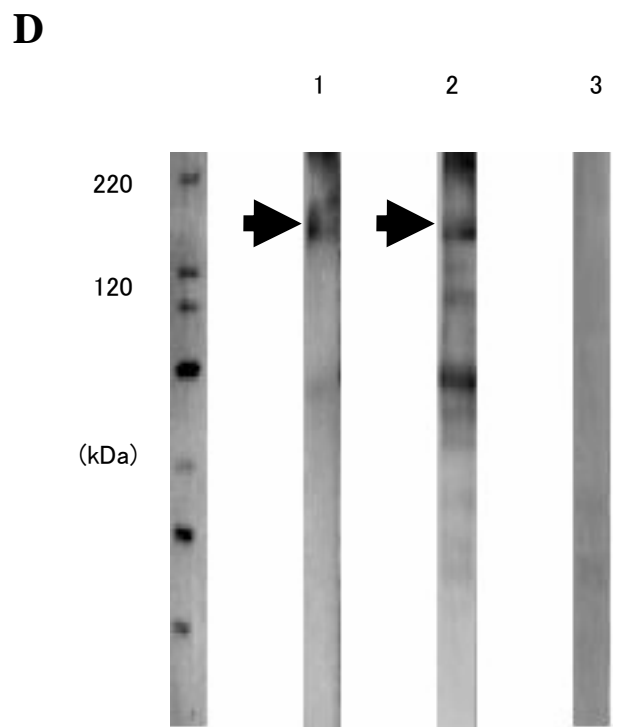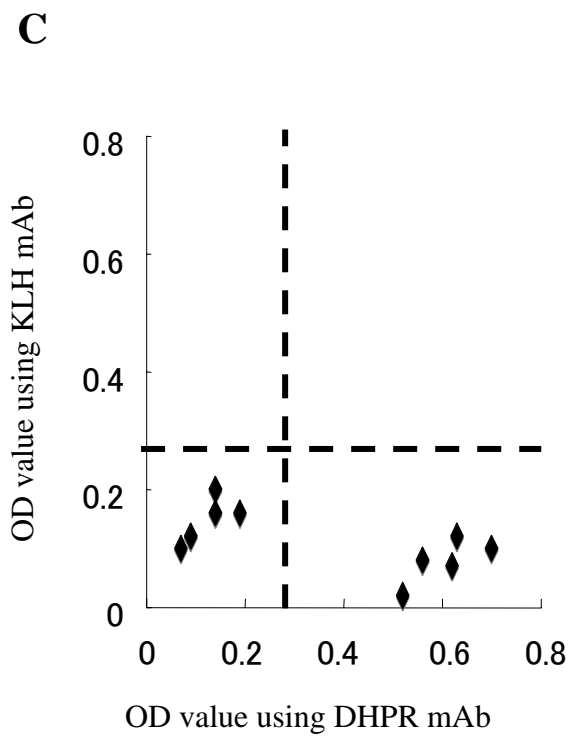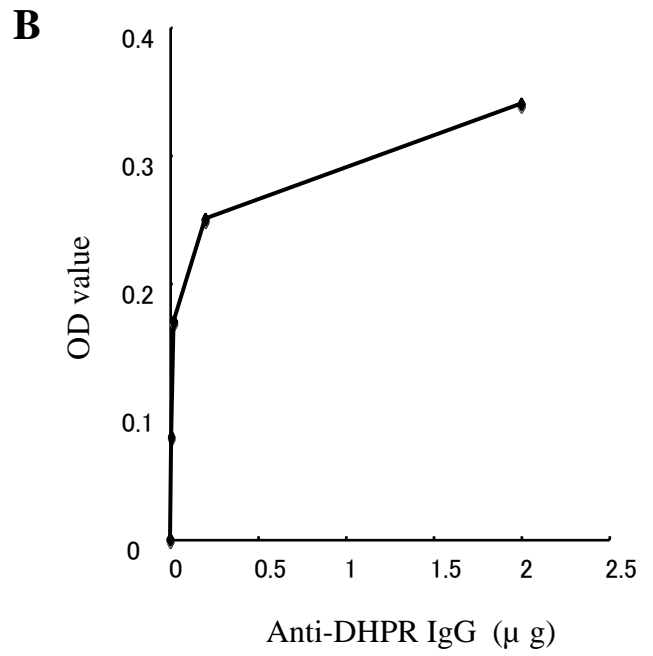DHPR Ab; autoantibody to DHPR, MG; myasthenia gravis, T-MG; MG with thymoma.

Figure 1

DHPR Ab (O.D.)

0.7
0.6
0.5
0.4
0.3
0.2
0.1
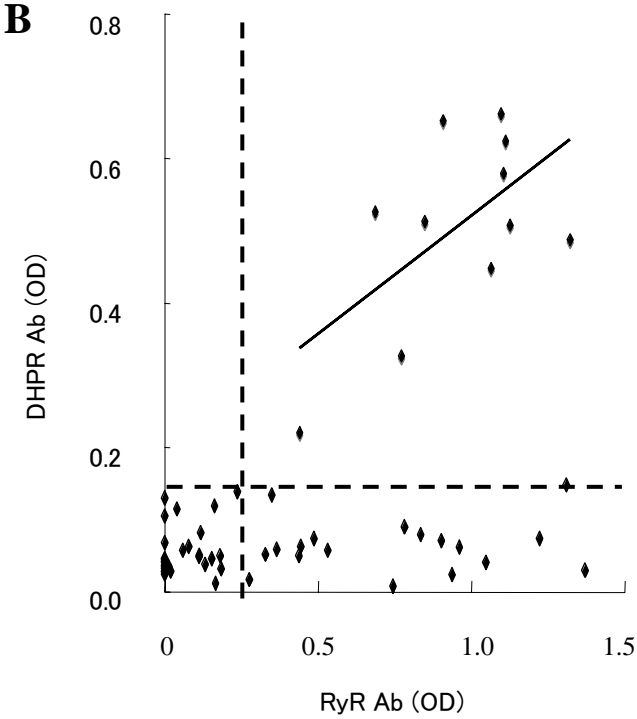0

T-MG   N-MG   LEMS   DC   NC
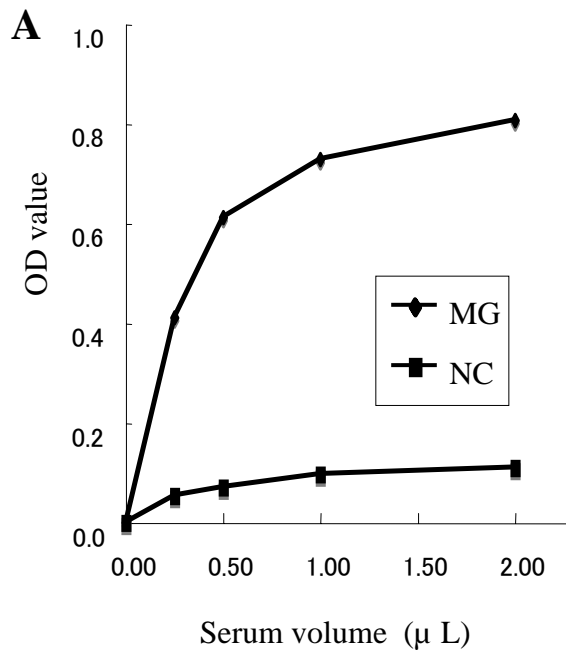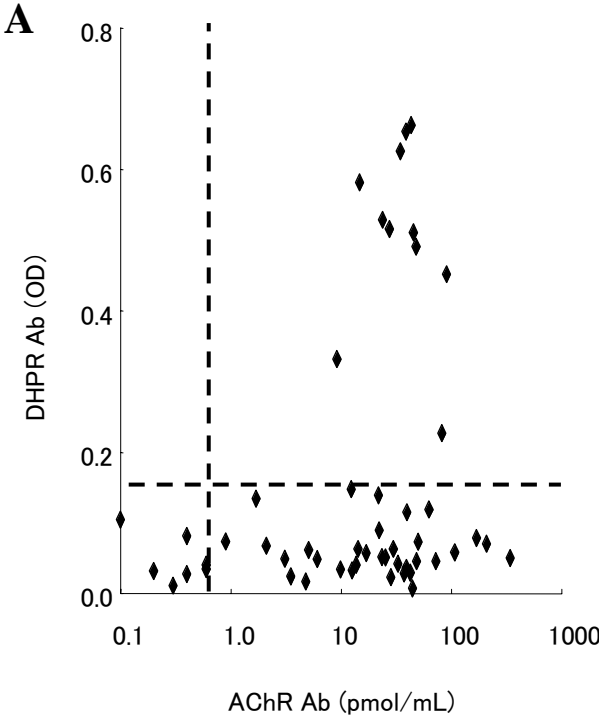
Figure 2

Figure 3

Figure 4
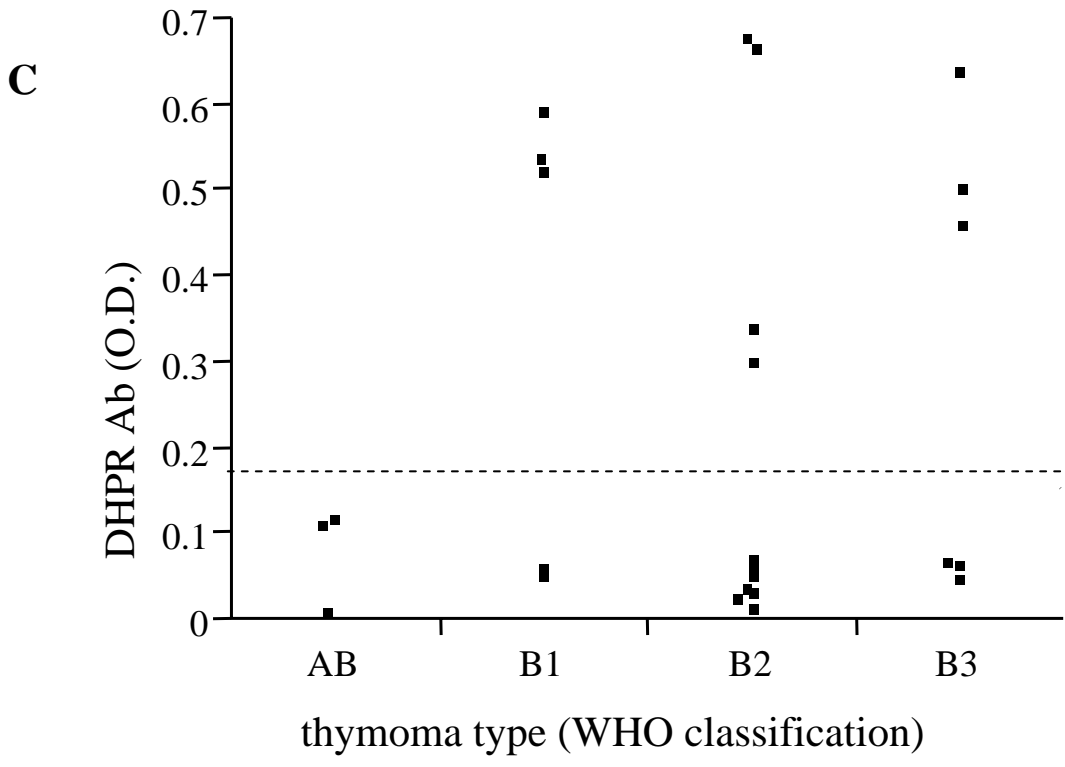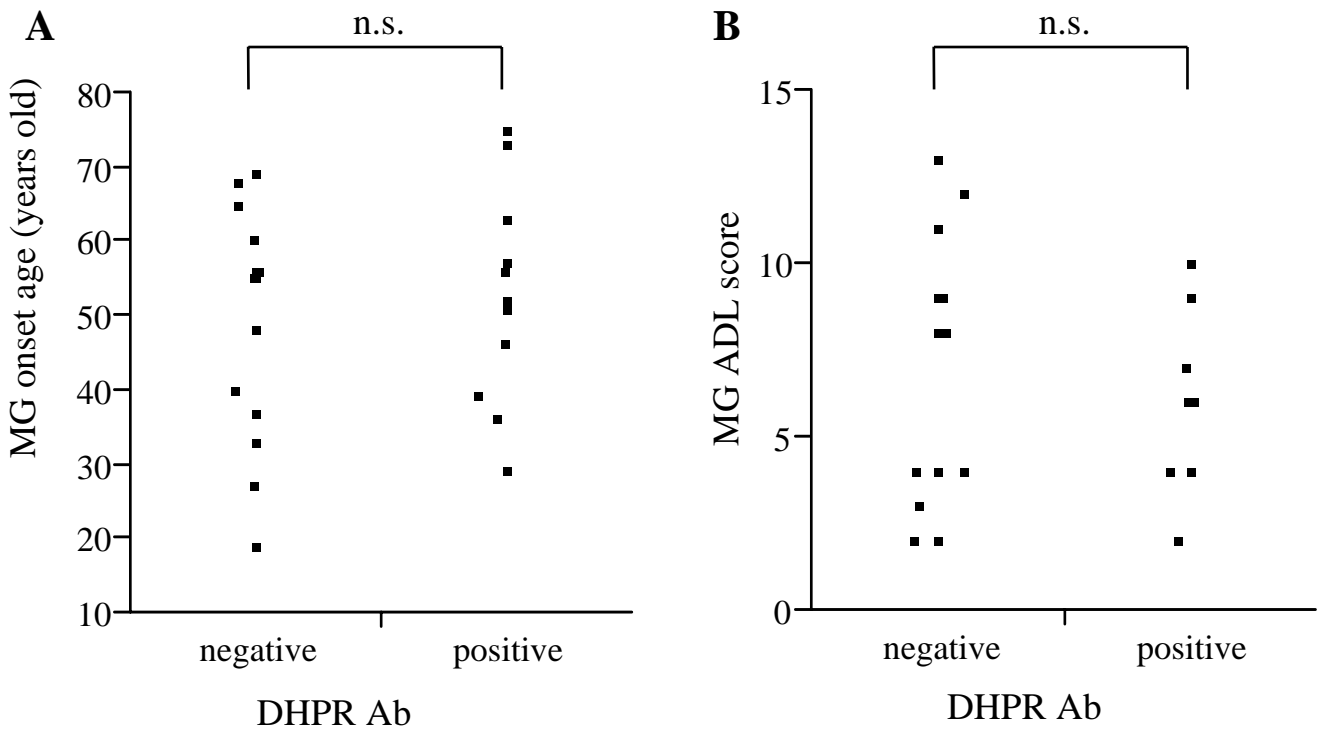
Table. Positive rate of DHPR Ab and RyR Ab

	Positive rate	
	DHPR Ab (%)	RyR Ab (%)
	p<0.01	
Myasthenia gravis with thymoma (T-MG, n=30)	37 *	70 *
Female patients with T-MG (n=18)	50] n.s.	83] n.s.
Male patients with T-MG (n=12)	17	50
Ocular type T-MG (n=7)	14] n.s.	22] n.s.
General type T-MG (n=23)	44	68
T-MG with RyR-Ab (n=21)	52] p<0.01	
T-MG without RyR-Ab (n=9)	0	
T-MG with DHPR Ab (n=10)		100] p<0.01
T-MG without DHPR Ab (n=20)		55
	p<0.01	
Myasthenia gravis without thymoma (N-MG, n=27)	0	30
Lambert-Eaton myasthenic syndrome (LEMS, n=14)	0	0
Disease Control (DC, n=35)	0	0
Normal Control (NC, n=20)	0	0

*; $p < 0.01$, analyzed with N-MG, LEMS, DC or NC.
Fisher's exact probability test was used for analysis.
(n.s.; no significnt difference)
AChR-Ab; anti-acetylcholine receptor antibody.
RyR-Ab; anti-ryanodine receptor antibody.
DHPR-Ab; anti-dihydropyridine receptor antibody.