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Longitudinal epitope mapping in MuSK myasthenia gravis: implications for disease severity

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ABSTRACT

Muscle weakness in MuSK myasthenia gravis (MG) is caused predominantly by IgG4 antibodies which block MuSK signalling and destabilize neuromuscular junctions. We determined whether the binding pattern of MuSK IgG4 antibodies change throughout the disease course ("epitope spreading"), and affect disease severity or treatment responsiveness.

We mapped the MuSK epitopes of 255 longitudinal serum samples of 53 unique MuSK MG patients from three independent cohorts with ELISA.

Antibodies against the MuSK Iglike-1 domain determine disease severity. Epitope spreading outside this domain did not contribute to disease severity nor to pyridostigmine responsiveness. This provides a rationale for epitope specific treatment strategies.

1. Introduction

MuSK myasthenia gravis (MG) is caused by antibodies to the receptor tyrosine kinase MuSK at the neuromuscular junction (Klooster et al., 2012; McConville et al., 2004; Niks et al., 2008). Unique to the disease are the prevalent IgG4 MuSK antibodies that prevent MuSK-Lrp4 interactions in a complement-independent manner and lead to functional inhibition of the AChR clustering pathway (Huijbers et al., 2013; Koneczny et al., 2013; Mori et al., 2012). The extracellular domain of MuSK consists of three N-terminal Ig-like domains and a Frizzled-like domain (MuSK-Fz-like). Most patients carry antibodies to the Ig-like domain 1 (MuSK-Ig1), which contains residue I96 essential for MuSK-Lrp4 interaction (Zhang et al., 2011). Antibodies to MuSK-Ig1 are likely to inhibit either by physically obstructing MuSK-Lrp4 binding, or by changing the conformation of MuSK rendering it unable to interact with Lrp4 and other interacting proteins. Antibodies to the Ig-like 2 domain (MuSK-Ig2) and MuSK-Fz-like have also been described, but their role in the disease process is unclear (Huijbers et al., 2013; McConville et al., 2004; Ohta et al., 2007). Moreover, intermolecular epitope spreading has been reported involving antibodies against MuSK and Lrp4, AChR or agrin (Gasperi et al., 2014; Higuchi et al., 2011; Zhang et al., 2012). Intramolecular and intermolecular epitope spreading has previously been described in bullous pemphigus where it correlated with disease severity (Di et al., 2011). Whether this is the case for MuSK MG is not known. Responsiveness to treatment with acetylcholine esterase inhibitor (AChEi) varies in MuSK MG. In AChR MG this treatment results in improvement of the symptoms by preventing breakdown of A-Ch. Thirty–fifty percent of MuSK MG patients treated with AChEi experience cholinergic side effects, ranging from cramps to worsening of symptoms (Evoli and Padua, 2013). The AChE-ColQ complex is stabilized in the neuromuscular junction by interactions with MuSK and could be blocked by MuSK antibodies (Kawakami et al., 2011). Therefore, we hypothesised that increased AChEi sensitivity might be correlated with a specific epitope pattern of MuSK antibodies (Cartaud et al., 2004; Otsuka et al., 2015).

Abbreviations: AChR, Acetylcholine receptor; DSS, Disease severity score; MuSK-Ig1, MuSK Ig-like 1 domain; Fz-like domain, Frizzled-like domain; LEMS Lambert-Eaton Myasthenic syndrome; Lrp4, Low-density lipoprotein receptor-related protein 4; MIR, Main immunogenic region; MuSK, Muscle specific kinase; MG, Myasthenia gravis; PV, Pemphigus Vulgaris; RIA, Radio-immunoprecipitation assay.

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To investigate epitope spreading and the association with disease severity, reactivity patterns and treatment responsiveness in MuSK MG, we mapped and independently confirmed the epitopes for a large set of (longitudinal) serum samples from 53 patients.

2. Patients and methods

2.1. Patient material

Patients were retrospectively selected based on clinical weakness typical for MuSK MG and a positive MuSK RIA assay (RSR Ltd., Cardiff, UK) and the availability of longitudinal serum samples. The patients were followed at the Leiden University Medical Centre (LUMC), the University Medical Centre Groningen, the Hospital Santa Creu i Sant Pau in Barcelona or the Università Cattolica del Sacro Cuore in Rome.

The control group consisted of six healthy individuals, eight patients with Lambert-Eaton myasthenic syndrome (LEMS), and nine patients with seronegative MG. All patients and controls gave written informed consent and the study was approved by the LUMC medical ethical committee.

Severity of symptoms was evaluated retrospectively by experienced neurologists (JV, JK, EN, and II) using the disease severity score (DSS) (Niks et al., 2008). Neurologists were blinded for MuSK antibody titres and used information from patients' charts to evaluate the severity of symptoms on the date of each serum sample.

2.2. Cloning of target genes and recombinant protein purification

The coding region of nine MuSK protein fragments was amplified from full length human *MuSK* cDNA using primers containing *Nde*I and *Xho*I restriction sites (Supplementary table 1). The *MuSK* containing inserts were *Nde*I and *Xho*I digested and cloned into the pET28a vector (EMD Biosciences, Novagen Brand, Madison, WI). All vectors were sequence verified and were used to produce partially overlapping recombinant MuSK protein fragments (Supplementary table 2).

Protein production was performed as described previously (Huijbers et al., 2013).

2.3. Epitope mapping MuSK ELISA

Insoluble protein fragments were diluted in 1 M urea and soluble protein fragments were diluted in PBS to a concentration of 3 μg/ml. 96-wells Maxisorp plates (Thermo Scientific, Nunc, Roskilde, Denmark) were coated with 100 μl diluted protein per well, and incubated overnight at 4 °C. After overnight incubation, the plate was processed as described previously (Huijbers et al., 2013).

Each ELISA experiment also included two negative control serum samples and one coating control per six plates to control for inter-plate and inter-experimental differences. As internal positive control, each plate contained a duplicate reactivity test for the full-length extracellular MuSK protein with a standard MuSK MG patient serum. All samples were tested in duplicate.

2.4. Statistical analysis

Each duplicate was averaged and corrected for the average PBS background signal. Each optical density value was next corrected for the internal positive control value. The 23 negative controls were used to determine the average background level. Signal detected in patients above this average background level plus three times the standard deviation were considered positive.

For statistical analysis the data was analysed using IBM SPSS statistics version 20 (SPSS Inc., Chicago, IL, U.S.A.). To assess the association between DSS and reactivity levels to MuSK-Ig1, taking into account the correlation between repeated measurements within patients, we fitted a linear mixed model with a fixed effect for the MuSK-Ig1 reactivity and random slopes and intercepts per patient. To address whether there was additional effect of reactivity against other domains on disease severity we entered them separately into the model together with the MuSK-Ig1 variable.

3. Results

3.1. Patient characteristics

To study epitope spreading in MuSK MG, 233 longitudinal serum samples of 20 Dutch and 11 Spanish patients were studied for their immunoreactivity against partially overlapping domains of human MuSK. Moreover, 22 samples of Italian patients were included to confirm our findings and study AChEi sensitivity in a separate cohort. Table 1 gives an overview of the demographic features of the patients included in this study. Mean age at onset was 42 years (49.2 in the Dutch population, 40.4 in the Spanish population, and 34.5 in the Italian cohort). The average age at onset in females was 8.9 years earlier compared to males although this difference was not significant ($p = 0.335$). Average follow-up for the Dutch patients was 6.1 years (1.0 to 19.2). Mean follow up among men was 6.5 yrs. (1.0–19.2) and for women 5.7 yrs. (1.5–11.1) with substantial variation between patients (Table 1, Fig. 1A).

3.2. Epitope spreading is uncommon in MuSK myasthenia gravis

We defined epitope spreading as: 'the occurrence of reactivity to other epitopes in any of the serum samples of a patient compared to the reactivity pattern in the first available serum sample of this patient'. All Dutch and Spanish patients ($n = 31$) showed reactivity to MuSK-Ig1 at the time of diagnosis. Sixteen patients showed additional reactivity to the MuSK-Ig2 and four patients had antibodies to the MuSK-Fz-like domain in the first available serum sample. In subsequent sera, epitope spreading was observed in 6 out of 31 patients accounting for 19% of MuSK MG patients tested (patients 1, 7, 11, 13, 18 and 31). When epitope spreading occurred, the majority of them developed reactivity against the MuSK-Fz-like domain (Fig. 1B, C). Three of these patients (7, 11, 13) already had reactivity against MuSK-Ig2 at the first time of examination, of which two (11 and 13) also had autoantibodies against the MuSK-Fz-like domain.

Of the patients who did not develop epitope spreading, 11 of 25 (44%) had only reactivity against MuSK-Ig1 (amino acids 21–125) whereas 48% also had reactivity against MuSK-Ig2 in their first available sample. Only two patients (8%) had reactivity against either the MuSK-Ig3 or the MuSK-Fz-like domain in addition to MuSK-Ig1 reactivity. None of the patients had reactivity against the intracellular domain at any point during their illness (data not shown).

Fig. 1 also illustrates the timing of the various treatments in the individual patients. As the treatment paradigms differed strongly between the patients it was not possible to statistically assess the effect of the treatments on reactivity against the different domains of MuSK. However, on the individual level the effects of treatment on antibody titres can be observed. Moreover, in five Italian patients, who went into remission, no reactivity against the MuSK-Ig1 domain could be detected, suggesting that these titres reflect their clinical status.

3.3. MuSK MG disease severity correlates with immunoreactivity against MuSK-Ig1 longitudinally

Since epitopes have been considered crucial determinants of the effectiveness and pathogenicity of an auto-immune response, we assessed whether reactivity against any domain of MuSK corresponded with the course of the disease and severity of the symptoms. A linear mixed effect model confirmed that reactivity against the N-terminal part of MuSK significantly correlates with DSS (combined cohorts: mean β-coefficient 0.159, $p < 0.000002$, Dutch cohort: β-coefficient 0.175, $p < 0.0001$, Spanish cohort: β-coefficient 0.107, $p < 0.036$). This