Anti-Lrp4 autoantibodies

Synonyms

- **L**ow density lipoprotein receptor-**r**elated protein-**4**
- MEGF7 (**m**ultiple epidermal growth factor [**EGF**]-like domain **7**)

Indications

- Myasthenia gravis
- Patients with suspected myasthenia and a negative test result as well for antibodies against acetylcholine receptors (anti-AChR) as for antibodies against muscle specific tyrosine kinase (anti-MuSK), so called serologically double negative patients.

see also

- Autoantibodies in diseases of neuromuscular transmission

Autoantibodies to the **l**ow density lipoprotein **r**eceptor-related **p**rotein **4** (anti-Lrp4), next to the antibodies against the acetylcholine receptor (anti-AChR) and to the antibodies against the muscle specific tyrosine kinase (anti-MuSK) may in future turn into the third specific marker antibody in the serological diagnosis of myasthenia gravis (MG). It seems that likewise to anti-AChR and anti-MuSK also anti-Lrp4 exerts its pathological role directly at the neuromuscular synapses. In this way an antibody mediated impairment of synapse functions could inhibit the signal transmission evoked by acetylcholine, provoking the myasthenic symptoms of muscle weakness. The demonstration of autoantibodies specific for myasthenia gravis, first anti-AChR (Simpson 1960; Lindsdrom et al. 1976) and 25 years later anti-MuSK (Hoch et al. 2001), by now has become an indispensable element in the serological diagnosis of myasthenia, in as much as the clinical diagnosis of this disease, especially in patents with mitigated symptoms, may run in difficulties. Since the sensitivity of the anti-AChR assays, used in routine screening, in cases of generalized myasthenia, amounts maximally 90 %, in more benign courses such as ocular myasthenia just maximally 50 %, and since antibodies against MuSK can be found at most in one half of the patients negative for anti-AChR, the common serological diagnosis fails at least in 10 % of the patients suffering from myasthenia (so called double negative forms). This serologic gap nowadays, owing to the discovery of the likewise disease specific antibodies to Lrp4, may become once more downscaled.

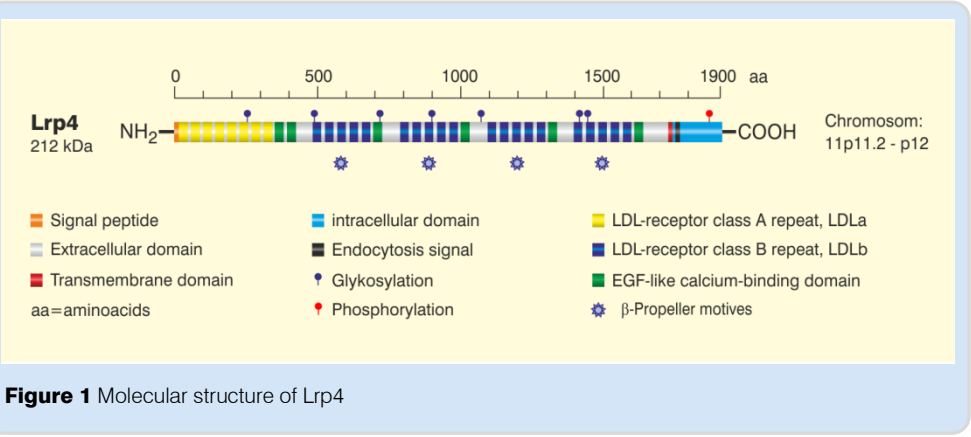

Figure 1 Molecular structure of Lrp4

Antigen

Lrp4 belongs to the family of low density lipoprotein (LDL) receptors. It constitutes a single-pass transmembrane protein (type 1) containing a relative large extracellular domain with multiple motives of LDL receptors, domains of calcium binding EGFs (epidermal growth factor) and β-propellers (figure 1), which form toroide like structures of four to eight circularly arranged β-sheets, each one containing four antiparallel loops.

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Lrp4, which is expressed in multiple tissues, is believed to exert important functions in the morphogenesis and ontogenesis of limbs, ectodermal organs, bones, lungs and kidneys (Johnson et al. 2005; Simon-Chazottes et al. 2006; Karner et al. 2010).

Lrp4, expressed within muscle tissues is essential for the development of the neuromuscular synapsis. Mutations of the *Lrp4*-gene impair substantially the development of the synapses (deficiencies, malformations) causing the animals decease as early as in the neonatal period. Phenotypically the defects are similar to those found in MuSK deficient mice.

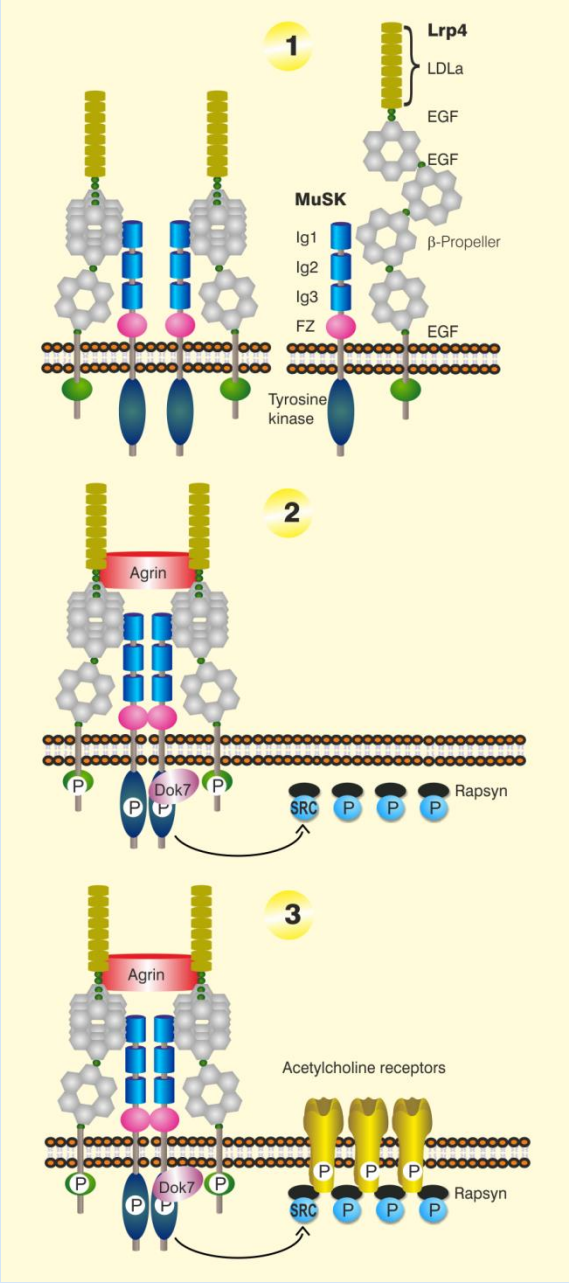

Figure 2

Simplified model of synaptogenesis at the neuromuscular junction.

[1] Interaction of Lrp4 and MuSK: Lrp4 interacts with the first immunoglobulin-domain (Ig1) of MuSK, involving the last five N-terminal LDLa receptor motives, the first two N-terminal EGF- and the first three β-propeller motives. This interaction may take place also in the absence of agrin.

[2] The interaction of Lrp4 with Agrin considerably enhances the MuSK-activating properties of Lrp4. The ligation of Agrin and Lrp4 involves the two last N-terminal LDLaR receptor motives, the following two EGF motives and the first N-terminal β-propeller motive (Zhang et al. 2011). The subsequent phosphorylation of cytoplasmatic tyrosine residues in Lpr4 and MuSK, the dimerisation and enhanced activation of MuSK subsequently enables the recruitment and the activation of the muscle specific adapter protein Dok7 by MuSK. The provision and phosphorylation of Dok7 promotes the transcription and accumulation of postsynaptic proteins. Using several different signal transduction pathways activated MuSK induces the aggregation and activation of rapsyn in the postsynaptic membrane.

[3] Activated rapsyn ligates to acetylcholine receptors and mediates their orthotope clustering within the subsynaptic membrane.

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In skeletal muscles Lrp4 acts as ligand of the muscle receptor specific tyrosine kinase (MuSK), which exerts a key role in synaptogenesis. The interaction of Lrp4 with MuSK and the hereby triggered phosphorylation and activation of MuSK are crucially modulated by the neuronal agrin, which in this context functions as allosteric regulator of Lrp4 (Zhang, B. et al. 2008; Zhang, W. et al. 2011). Lrp4 has been identified as the connecting link of the agrin-MuSK signal transduction pathway (figure 2), long searched for and formerly provisionally named myotube-associated specifity component (MASC). During mygenesis Lrp4 is expressed together with MuSK in myotubes, In the adult skeletal muscle the transcription of the *Lrp4*-gene takes place close by the subsynaptic myonuclei.

Yet in the non-innervated myotubes, i. e. in the absence of agrin, Lrp4 is able to interact with and to activate MuSK, thus initiating the essential steps of signal transduction, which in turn regulate the clustering of acetylcholine receptors in the region of the future synapses (prepattering). As soon as the neurite of the motoneuron will join this site of receptor clustering, agrin synthesized by motoneurons will be released. Agrin enters into the subsynaptic membrane where it interacts with Lrp4, stimulating its phosphorylation. The binding of Agrin enables Lrp4 to bind and to activate MuSK more effectively. Agrin itself does not ligate MuSK directly but requires Lrp4 as mediator. Activated MuSK mediates the clustering of acetylcholine receptors in the synapses and plays in this way a key role in the formation, maintenance and repair of the neuromuscular synapses (Apel et al. 1997; Glass et al. 1996; Zhang et al. 2008; Kim et al. 2008).

Autoantibodies

Autoantibodies to Lrp4 recognize the extracellular domain of the molecule and presumably bind to conformational epitopes. Fixation of culture cells expressing Lrp4 on their cell membranes abolishes the binding of human autoantibodies but not the reaction with rabbit antibodies synthesized against non-conformal epitopes. The antibodies may possibly recognize and block the Lprp4 binding sites for agrin (figure 2), because of their capacity to inhibit the ligation between agrin and Lrp4 *in vitro* (Higuchi et al. 2011). The antibodies, as far as previous investigations have shown, belong to the immunoglobulin subclass IgG1, and are therefore able to activate the complement system.

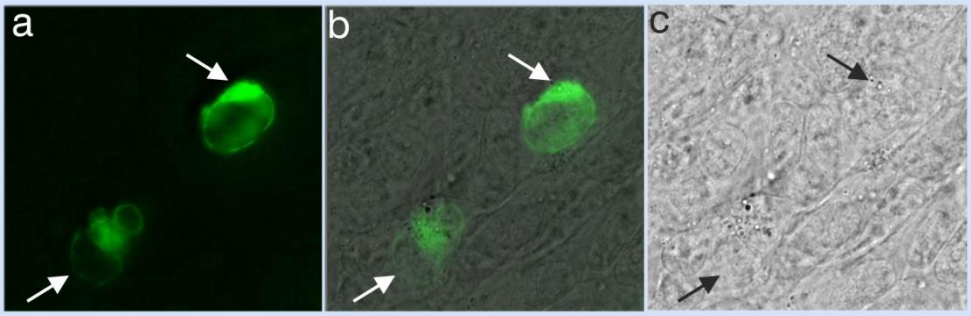

Figure 3 Human embryonic kidney cells (HEK293) transiently transfected with LRP4-GFP-cDNA express LRP4 on the cell membrane and within the cytoplasm. The fluorescence microscopy picture (a) depicts only the green fluorescing LRP4-GFP expressing cells, the non-transfected and non-expressing residual cells seen in phase microscopy (c) do not exhibit green fluorescence. b: Overlay of a and c. Objective magnification: 40-fold.

Immunopathology

The pathological role of anti-Lrp4 in the emergence of myasthenic symptoms in humans is not clarified yet. Following the hitherto known experimental data, one can assume, that the antibodies are directly involved in the development of the neurological lesions and do not constitute a mere epiphenomenon of the disease. The overwhelming part of the Lrp4 molecule is located on the extracellular site of the plasma membrane, making it therefore accessible for the attack of autoantibodies also *in vivo*.

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Once bound, anti-Lrp4 autoantibodies may inhibit the ligation of agrin to Lrp4 and thus impair the synaptogenesis and block the neuromuscular signal transduction pathway. As could be shown by *in vitro* experiments anti-Lrp4 inhibited the biding of agrin to Lrp4 (Higuchi et al. 2011).

The antibodies also inhibited in a dosage dependent manor the clustering of acetylcholine receptors in cultured myotubes, which is a necessary prerequisite for the synaptogenesis (Pevzner et al. 2011; Zhang et al. 2012). It may be discussed if the binding of the antibodies to Lrp4 not only hampers the ligation of agrin but also the activation of MuSK. Furthermore it may be conceivable that Lrp4 bound autoantibodies stimulate its internalization into the cell and thus reduce its extracellular quota. Human anti-Lrp4 autoantibodies, belonging to the subclass IgG1, probably may destruct the receptors in a complement dependent manner. Since at the moment the function of Lrp4 in the adult muscle is not clarified in detail, the pathological role of the antibodies cannot be conclusively assessed.

Prevalence

The prevalence of anti-Lrp4 in double serological negative patients with myasthenia (negative for anti-AChR and anti-MuSK) has been evaluated in three research studies (table 1). The communicated mean prevalence of ant-Lrp4 in these studies was 3,3 %, 9,2 % and 50 % respectively. The considerable divergence of the results may be attributable in first line to variations in the sensitivity of the different screening tests used. Also ethnical influences and environmental factors me be considered .But as far as the classification of persons as being negative for anti-AChR is concerned, it should be mentioned, that the anti-AChR assays used in routine testing are incapable to detect all seropositive patients.

Tabel 1 Prevalence of anti-Lrp4 autoantibodies [%] in patients with myasthenia gravis, other neurological diseases and healthy persons.

Autor	Higuchi (2011)		Pevzner (2011)		Zhang (2012)		Seelig (2013)[◊]	
Disease	n	[%]	n	[%]	n	[%]	n	[%]
Myasthenia								
a-AChR – a-MuSK –	272	**3,3**	38	**50,0**	120	**9,2**		
a-AChR + a-MuSK –	100	**0,0**			61	**0,0**		
a-AChR – a-MuSK +	28	**10,7**	11	**9,2**	36	**2,8**	33	**3,0**
Lambert-Eaton-syndrome	101	**1,0**						
Neuromyelitis optica					16	**12,5**		
Neurological diseases *					60	**0,0**		
Healthe persons	100	**0,0**	4	**0,0**	45	**0,0**		

a-AChR:anti-AChR — a-MuSK: anti-MuSK.
AChR: acetylcholine receptor — MuSK: muscle specific tyrosine kinase.

n: number; +/–: patients positive or negative fort he indicated antibody.

* Psychiatric diseases (10), various neurological diseases (41), amyotrophic lateral sclerosis (9).
◊ own unpublished results.

Using more sensitive AChR-assay, with acetylcholine-receptor transfected cells, revealing an augmented receptor density (i. e. antigen concentration) on the cell membrane, it could be shown that some of the formerly anti-AChR negative ("serological negative") patients indeed harbored antibodies to acetylcholine receptors (Leite et al. 2008). We have therefore to consid-

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er that some of the so called anti-AChR-negative patients with positive anti-Lrp4-test in truth could turn out to belong to a subgroup harboring very low anti-AChR antibody concentrations, not demonstrably in routine assays ("false serological negatives").

Antibodies to Lrp4 were also detected in 2,8 - 10,7 % of anti-AChR negative but anti-MuSK positive myasthenia patients (table 1). In a preliminary study we found anti-Lrp4 in three anti-MuSK positive patients by means of an immunoprecipitation assay using cell lysates of transiently transfected HEK293-cells, a result, which, however, could be confirmed only for one of the patients by an indirect immunofluorescence test with transfected HEK293 cells or by line blot using a native, recombinant extracellular domain of Lrp4 (figure 4, 5, 6).

None of the three studies revealed antibodies to Lrp4 neither in healthy persons (n = 149) nor in patients with myasthenia harboring antibodies to acetylcholine receptors (n = 161), tested by conventional assays.

No anti-Lrp4 was detected in the above mentioned studies in 76 patients with neurological and psychiatric diseases (amyotrophic lateral sclerosis, schizophrenia, multiple sclerosis, Guillain-Barré syndrome etc.). Of 101 patients with Lambert-Eaton myasthenic syndrome there was one patient harboring anti-Lrp4 (Zang B et al. 2012).

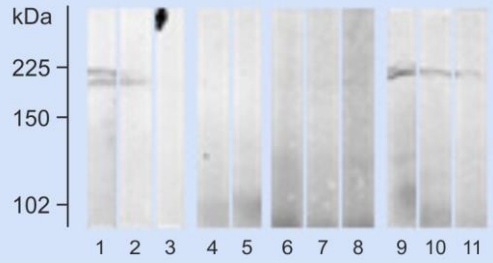

Figure 4
Immunoprecipitation of lysates of LRP4-GFP transfected HEK293 cells by anti-human LRP4 from rabbit (1-3), anti-MuSK negative, anti-AChR positive (4 - 8) and anti-MuSK positive (9 - 11) patient sera. SDS-PAGE of precipitated proteins. Demonstration of precipitated LRP4 by western blot using rabbit anti-human LRP4 and peroxidase labeled anti-rabbit IgG as secondary antibody. The presence of anti-Lrp4 in 10 and 11could not be confirmed by IIFT and line blot assay.

Since the sera of two of 16 patients (12,5 %) suffering from neuromyelitis optica (NMO) revealed anti-Lrp4 in concentrations corresponding to that of myasthenic patients one may speculate from the statistical point of view, that as shown in the study of Zhang and coworkers (2012) the prevalence of anti-Lrp4 in NMO (12,5 %) indeed may be higher than in the contingent of double serological negative patients with myasthenia (9,2 %). However, it should be considered, that only 16 patients with NMO were screened, and that no data were given concerning their clinical and serological status. One should also bear in mind the possible association of NMO and myasthenia, coincidences that have been repeatedly noted (McKeon et al. 2009; Vaknin-Dembinsky et al. 2011; Jarius et al. 2012). As far as the marker antibodies of the two diseases were concerned (anti-Aquaporin 4 [NMO], anti-AChR [MG]), all combinatorial possibilities were found. There were mainly patients positive for both anti-Aquaporin 4 and anti-AChR, but there were also patients in whom only the one or the other antibody was present. For clarification the prevalence of anti-Lrp4 has to be evaluated in detail in a larger study group.

Clinic

In contrast to the findings in seropositive patients (anti-AChR) with myasthenia there are no reports of associations with thymoma. The predominance of female patients in anti-Lrp4 positives (Pevzner et al. 2011) also has to be confirmed in a larger patient group.

Test methods

For detection of anti-Lrp4 in patients sera various screening methods were applied:

- indirect immunofluorescence test (figure 5) using HEK293-cells transiently transfected either with Lrp4-cDNA (Higuchi et al, 2011) or transiently transfected by means of an bicistronic vector, coding for full length of human Lrp4 and an enhanced green fluorescent protein

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(EGFP) (Pevzner et al. 2011), or transiently transfected with an pcDNA 3.1/ CT-GFP-TOPO®-vector, coding the full length human Lrp4 linked to the coding sequence of a green fluorescent protein (Lrp4-GFP), which will be expressed only in the presence of correctly orientated cDNA of Lrp4 (own investigations).

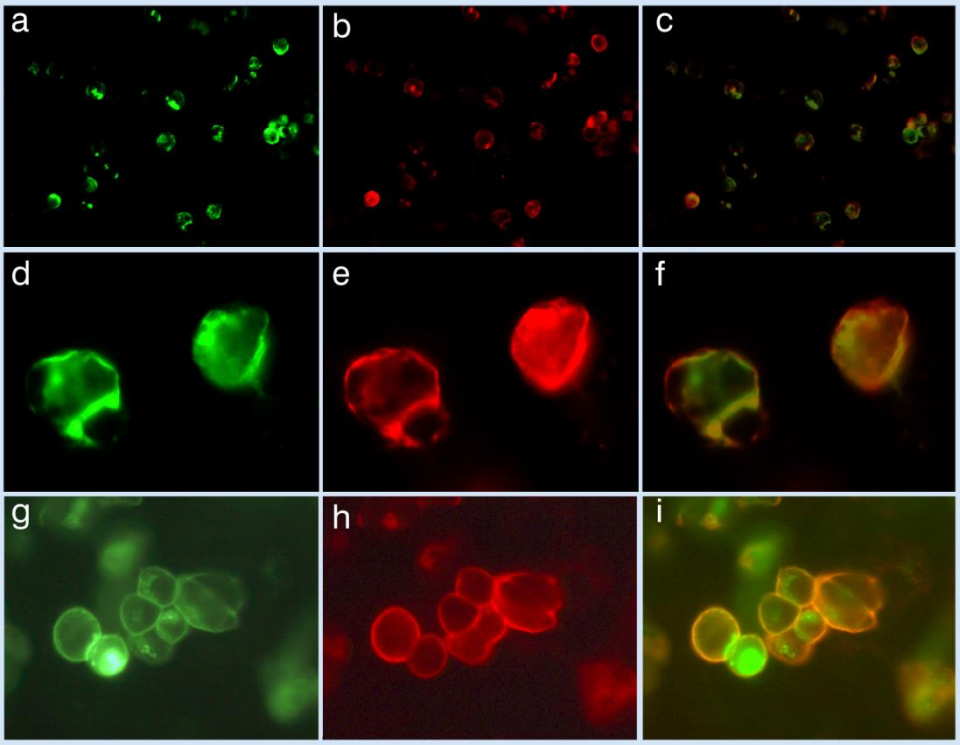

Figure 5 Demonstration of antibodies against LRP4 by means of indirect immunofluorescence. Transiently transfected HEK293 cells incubated with anit-LRP4 (antibody reacting with the extracellular domain) from rabbit (a - f) and with the serum of an anti-MusK positive patient with myasthenia gravis (g - i). Bound antibodies were visualized with rhodamine labeled anti-rabbit IgG or Alexa Fluor 568 labeled anti-human IgG respectively. In a, d and g is shown the intrinsic green fluorescence of LRP4-GFP expressing cells. b and e: Demonstration of bound anti-LRP4 (rabbit) with rhodamine labeled secondary antibody; h: visualization of bound human anti-Lrp4 using Alexa Fluor labeled anti-human IgG. c, f, i: respective overlays. Objective magnifications: a - c: 10-fold; d -f: 40-fold, g - i 20-fold.

- Lineblot using the native recombinant extracellular domain of Lrp4 obtained as secreted product of permanently transfected CHO cells (modified vector pSecTag2AzL; own investigations; figure 6).
- Luminescence assay using as antigen Lrp4 coupled to Gaussia-luziferase, synthesized in HEK293-cells (Higuchi et al. 2011).
- Immunoprecipitation assay using either Lrp4 extracted from transiently transfected HEK293-cells (Higuchi et al. 2011, Zhang et al. 2012) or Lrp4-GFP extracted from transiently transfected HEK293-cells or the native recombinant extracellular domain of Lrp4 obtained from permanently transfected protein secreting CHO-cells (figure 4; own investigations).
- Elisa using the extracellular domain of Lrp4, synthesized in HEK293-cells (Zhang et al. 2012).

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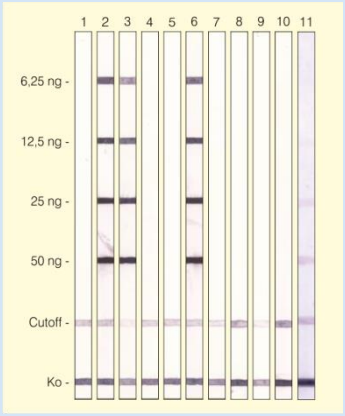

Figure 6 Semiquantitative linebot for screening of anti-Lrp4 autoantibodies.

The complete His-tagged extracellular domain of Lrp4 was synthetized in permanently transfected CHO cells (using a modified pSecTag2A vector, Invitrogen), two times purified by Ni^{2+}-affinity chromatography, dialyzed and sprayed on nitrocellulose in concentrations [ng/mL] displayed on the ordinate (SX sciDROP NANO, Scienion AG).

1: negative control; 2, 3: positive controls (anti-Lrp4 from rabbit, Sigma), 4 - 10: sera of patients with anti-AChR negative, anti-MuSK positve myasthenia gravis, one of whom (6) harboring anti-Lrp4. 11: seronegative (anti-AChR, anti-MuSK) patient with myasthenia showing an equivocal result, which could not be confirmed by IIFT, possibly due to a very concentration of anti-Lrp4.

Ko: function control (anti-human-IgG)

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