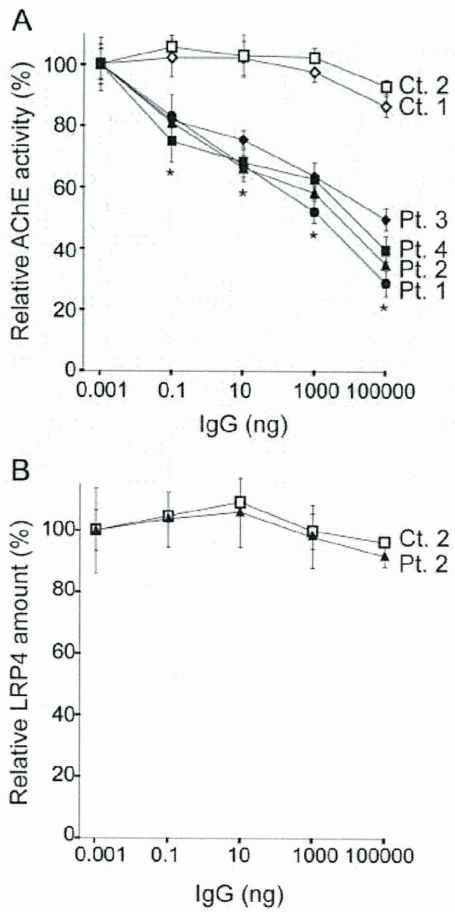
Figure 3 In vitro plate-binding assays

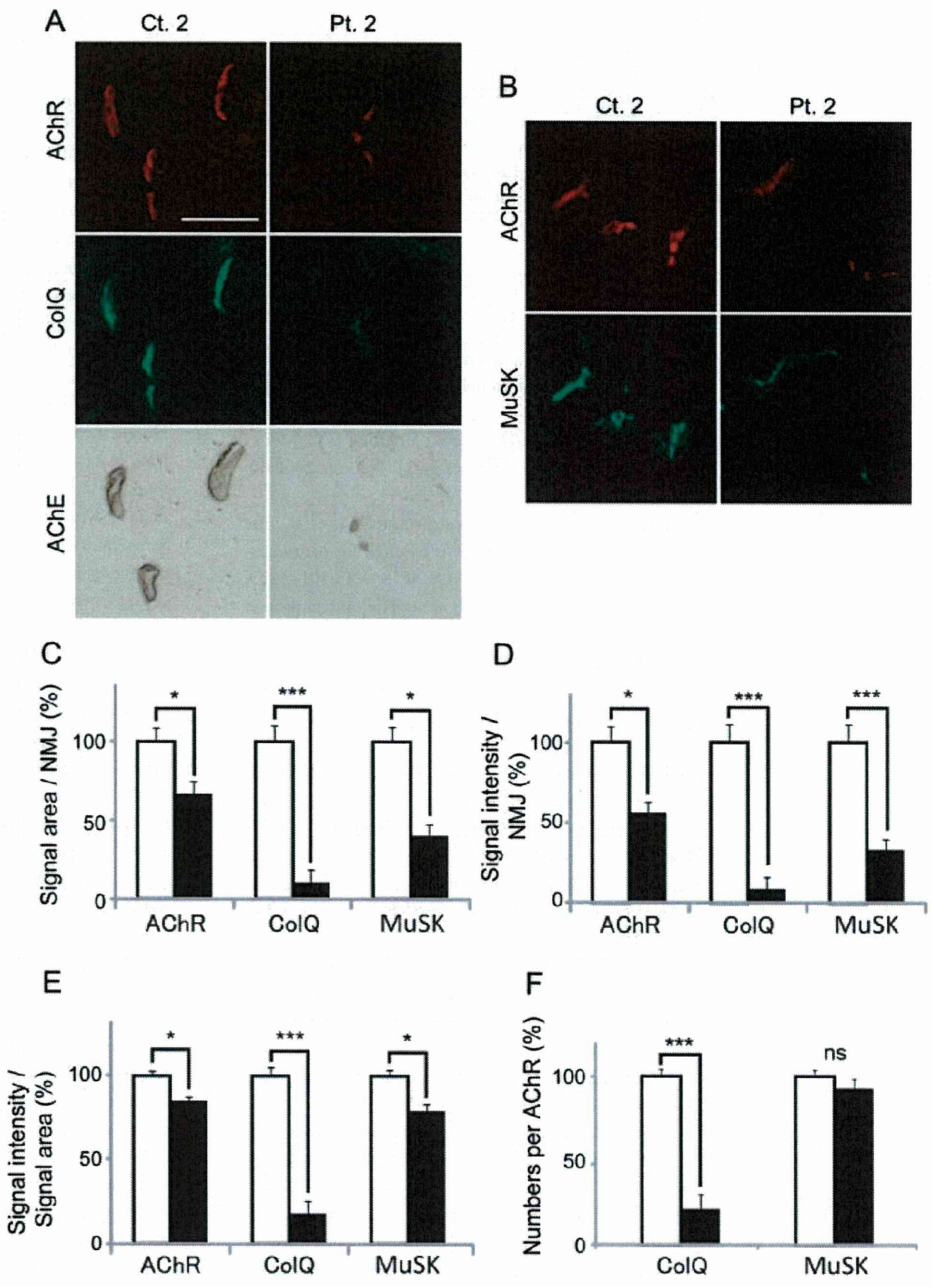


(A) Increasing amounts of muscle-specific receptor tyrosine kinase (MuSK)–immunoglobulin G (IgG) block binding of the purified recombinant collagen Q (ColQ)-tailed acetylcholinesterase (AChE) to the extracellular domain of human MuSK that is coated on a 96-well plate. Bound ColQ-tailed AChE is quantified by AChE activity. AChE activities are normalized for that at 1 pg IgG of each sample. Mean and SEM of 3 experiments are plotted. *$p < 0.01$ between controls and patients. (B) MuSK-IgG does not block binding of the purified FLAG-tagged extracellular domain of human LRP4 (LRP4N-FLAG) to MuSK that is coated on a 96-well plate. Bound LRP4N-FLAG is quantified with anti-FLAG-HRP. HRP activities are normalized for that at 1 pg IgG of each sample. Mean and SEM of 3 experiments are plotted.

NMJ AChR deficiency. Similarly, injection of MuSK-IgG into mice reduces the number of AChRs at the NMJ to 22% of controls, compromises the apposition of the presynaptic and postsynaptic components of the NMJ,[24] and reduces muscle contractility.[27] A recent report demonstrates that MuSK-IgG enhances internalization of MuSK from plasma membrane, which leads to progressive dispersal of postsynaptic AChRs by disruption of the MuSK scaffold and not by disruption of the agrin/LRP4/MuSK signaling pathway.[26] To summarize, MuSK-IgG does not reduce AChR expression in cultured cells, but active and passive immunization of model animals results in AChR deficiency, which is not likely due to blocking of the agrin/LRP4/MuSK pathway. Our findings that MuSK-IgG blocks binding of ColQ but not of LRP4 to MuSK are consistent with these findings. In myotubes of *Colq*−/− mice, the number of membrane-bound MuSK is prominently reduced, and agrin-mediated phosphorylation of the AChR β subunit and the subsequent clustering of AChR are reduced to 30%–50% of the wild type.[32] Thus, compromised clustering of AChRs at the NMJs in some MuSK-MG patients could result from blocking of ColQ binding to MuSK but not from blocking of LRP4 binding to MuSK.

Although our results predict endplate AChE deficiency in MuSK-MG patients, we found no AChE deficiency in intercostal muscles of one reported[33] and two unreported cases of MuSK-MG. In vitro microelectrode studies showed a normal EPP decay time constant.[34] In the 3 MuSK-MG patients observed by us, the MEPC decay times were shorter than normal, normal, and 2-fold prolonged[33] compared to controls. Thus, our biopsy findings do not indicate that MuSK-MG patients have endplate AChE deficiency. There are 2 plausible explanations for the apparently contradicting observation on the human biopsies and the in vitro and in vivo studies. First, MuSK-IgG does not block binding of ColQ-tailed AChE to the NMJ to a detectable extent in the patients. ColQ is localized to the synaptic basal lamina via 2 mechanisms: one is by binding to heparin sulfate proteoglycans including perlecan,[7,8] and the other is by binding to MuSK.[9] We previously reported that both mechanisms are required for in vitro anchoring of human ColQ to the frog NMJ.[23] Reduced clustering of ColQ in our passive transfer model suggests that ColQ needs to bind to at least MuSK in mice. However, binding of ColQ to MuSK is dispensable for clustering ColQ in humans, but is required for facilitating AChR clustering.[32] Second, AChE could be deficient in severely affected muscles but not in the biopsied intercostal muscles. However, the respiratory functions of the patients who had intercostal muscle biopsies were severely compromised. Expression levels of MuSK[35] and ColQ[36] were reported to be different between slow- and fast-twitch muscles in model animals. In active[37] and passive[26] immunization models, slow-twitch diaphragm was more severely affected than fast-twitch tibialis anterior and intercostal muscles. Similar uneven distributions of affected muscles are reported in MuSK-MG patients.[12] Further studies will be required to elucidate the basis of the discrepant observations between mice and humans.

Figure 4 Passive transfer of muscle-specific receptor tyrosine kinase (MuSK)–immunoglobulin G (IgG) of control 2 and patient 2 to C57BL/6J mice



(A, B) Quadriceps muscle sections of mice injected with IgG of control 2 or patient 2 are stained for acetylcholine receptor (AChR) by Alexa594-labeled α-bungarotoxin, collagen Q (ColQ) and MuSK by immunostaining, acetylcholinesterase (AChE) by cytochemical staining. Scale bar = 40 μm. Signal areas (C), intensities (D), and densities (intensity/area) (E) of the indicted molecules per neuromuscular junction (NMJ) are shown in mean and SEM. (F) Densities of ColQ and MuSK are normalized for the density of AChR to estimate the number of ColQ and MuSK per AChR. For AChR, ColQ, and MuSK, we analyzed 44 NMJs of control 2 and 23 NMJs of patient 2. For MuSK, we analyzed 82 NMJs of control 2 and 42 NMJs of patient 2. Areas and intensities are quantified by the BZ-9000 microscope (Keyence). Open and closed bars represent control 2 and patient 2, respectively. $^{*}p < 0.05$, $^{***}p < 0.001$. NS = not significant.

AUTHOR CONTRIBUTIONS

Dr. Kawakami designed and conducted experiments and wrote the paper. Dr. Ito designed and conducted experiments. Dr. Hirayama conducted experiments. Dr. Sahashi diagnosed a patient and conceived the study. Dr. Ohkawara designed experiments. Dr. Masuda designed experiments. Dr. Nishida diagnosed a patient and conceived the study. Dr. Mabuchi diagnosed a patient and conceived the study. Dr. Engel diagnosed a patient, conceived studies, designed experiments, and wrote the paper. Dr. Ohno conceived study, designed experiments, and wrote the paper.

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DISCLOSURE

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