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Anti-LGI4 Antibody Is a Novel Juxtaparanodal Autoantibody for Chronic Inflammatory Demyelinating Polyneuropathy

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Abstract

Background and Objectives

The objective of this study was to discover novel nodal autoantibodies in chronic inflammatory demyelinating polyneuropathy (CIDP).

Methods

We screened for autoantibodies that bind to mouse sciatic nerves and dorsal root ganglia (DRG) using indirect immunofluorescence (IFA) assays with sera from 113 patients with CIDP seronegative for anti-neurofascin 155 and anticontactin-1 antibodies and 127 controls. Western blotting, IFA assays using HEK293T cells transfected with relevant antigen expression plasmids, and cell-based RNA interference assays were used to identify target antigens. *Krox20* and *Periaxin* expression, both of which independently control peripheral nerve myelination, was assessed by quantitative real-time PCR after application of patient and control sera to Schwann cells.

Results

Sera from 4 patients with CIDP, but not control sera, selectively bound to the nodal regions of sciatic nerves and DRG satellite glia ($p = 0.048$). The main immunoglobulin G (IgG) subtype was IgG4. IgG from these 4 patients stained a 60-kDa band on Western blots of mouse DRG and sciatic nerve lysates. These features indicated leucine-rich repeat LGI family member 4 (LGI4) as a candidate antigen. A commercial anti-LGI4 antibody and IgG from all 4 seropositive patients with CIDP showed the same immunostaining patterns of DRG and cultured rat Schwann cells and bound to the 60-kDa protein in Western blots of LGI4 overexpression lysates. IgG from 3 seropositive patients, but none from controls, bound to cells cotransfected with plasmids containing LGI4 and a disintegrin and metalloprotease domain–containing protein 22 (ADAM22), an LGI4 receptor. In cultured rat Schwann and human melanoma cells constitutively expressing LGI4, *LGI4* siRNA effectively downregulated *LGI4* and reduced patients' IgG binding compared with scrambled siRNA. Application of serum from a positive patient to Schwann cells expressing ADAM22 significantly reduced the expression of *Krox20*, but not *Periaxin*. Anti-LGI4 antibody-positive patients had a relatively old age at onset (mean age 58 years), motor weakness, deep and superficial sensory impairment with Romberg sign, and extremely high levels of CSF protein. Three patients showed subacute CIDP onset resembling Guillain-Barré syndrome.

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Glossary

CASPR1 = contactin-associated protein 1; **CIDP** = chronic inflammatory demyelinating polyneuropathy; **IFA** = indirect immunofluorescence; **IgG** = immunoglobulin G; **IVIg** = IV immunoglobulin; **LGI4** = LGI family member 4; **MFI** = mean fluorescence intensity; **PBS** = phosphate-buffered saline; **RT** = room temperature; **WB** = Western blotting.

Discussion

IgG4 anti-LGI4 antibodies are found in some elderly patients with CIDP who present subacute sensory impairment and motor weakness and are worth measuring, particularly in patients with symptoms resembling Guillain-Barré syndrome.

Chronic inflammatory demyelinating polyneuropathy (CIDP), an acquired immune-mediated disorder affecting the peripheral nerves, is now recognized as a syndrome because of its clinical and pathologic heterogeneity.[1] Although it is widely accepted that this disorder has an autoimmune pathogenesis, the relevant autoantigens and exact underlying mechanisms remain elusive.[2] Recently, autoantibodies against proteins located around the nodes of Ranvier, such as neurofascin 155 (NF155), contactin-1 (CNTN1), and contactin-associated protein 1 (CASPR1), have been discovered in subsets of patients with CIDP.[3-7] Immunoglobulin G (IgG) subclass analysis of these antibodies revealed predominant elevation of the IgG4 subclass.[3-7]

IgG4 antibodies against nodal/paranodal proteins are associated with unique features; therefore, CIDPs with these antibodies are now collectively termed autoimmune nodopathies.[8] For example, compared with seronegative patients with CIDP, anti-NF155 antibody-positive ($NF155^+$) CIDP shows a younger age at onset, a mostly chronic progressive course but subacute onset in some (38% in one study),[3] distal dominant sensorimotor neuropathy, deep sensory impairment leading to sensory ataxia, tremor, extremely high CSF protein levels, symmetric spinal root and plexus hypertrophy, frequent occurrence of subclinical demyelination in the trigeminal and facial nerves and the optic nerve, and poor response to IV immunoglobulin (IVIg).[3-5,7-9] Furthermore, anti-CNTN1 antibody–positive ($CNTN1^+$) CIDP is associated with a relatively older age at onset, subacute onset in 35%–50% of cases, distal dominant sensorimotor neuropathy, proprioceptive impairment leading to sensory ataxia, distal muscle atrophy, and very high CSF protein levels.[6,10-12] However, some seronegative patients with CIDP also demonstrate features similar to those of autoimmune nodopathy. These observations prompted us to search for unknown autoantibodies and the relevant nodal antigens in these seronegative patients. The blood-nerve barrier is absent at dorsal root ganglia (DRG), providing accessibility to autoantibodies[13]; therefore, we surveyed the reactivity of sera from patients with CIDP against mouse DRG and sciatic nerves using a previously established tissue-based indirect immunofluorescence (IFA).[14] In this study, we report a novel IgG4 nodal autoantibody and the target antigen.

Methods

Participants

One hundred thirteen consecutive anti-NF155 and anti-CNTN1 antibody–negative patients with CIDP, 76 males and 37 females, from Kyushu University Hospital, were enrolled in this study. Most patients had been thoroughly examined for CIDP in the Department of Neurology at Kyushu University Hospital in 2001 or later, while some were referred to the department for anti-NF155 and anti-CNTN1 antibody assays in 2014 or later. All seronegative patients fulfilled the definite electrodiagnostic criteria of the European Federation of Neurologic Societies/Peripheral Nerve Society for the diagnosis of CIDP.[15] In addition, 127 controls, 45 males and 82 females, mean age 48.6 ± 17.8 years (range 13–87), were included. These included 35 healthy controls and 92 patients with other neurologic diseases who were examined at Kyushu University Hospital and Fukuoka Central Hospital (details in eTable 1, links.lww.com/NXI/A792).

Tissue-Based Immunofluorescence Assays

Lumbar DRG and sciatic nerves were removed from 10-week-old male C57BL/6 mice. DRG were fixed in 10% formalin and processed into 3-μm paraffin sections. Indirect IFAs were performed on these specimens using sera from patients (diluted 1:60) alone or together with commercial antileucine-rich repeat LGI family member 4 (LGI4) polyclonal antibodies (diluted 1:60) (Novus Biologicals, Centennial, CO), anti-S100β monoclonal antibodies (diluted 1:500) (Abcam, Cambridge, UK), antiglutamine synthetase polyclonal antibodies (diluted 1:500) (Invitrogen, Waltham, MA), or anti-CASPR1 polyclonal antibodies (diluted 1:500) (Abcam). Alexa 488-labeled antihuman IgG and Alexa 594-labeled antirabbit or mouse IgG antibodies (Thermo Fisher Scientific, Waltham, MA) were used as secondary antibodies (1:500 dilution). Sciatic nerves were dissected and fixed for 10 minutes in freshly prepared phosphate-buffered saline (PBS) containing 4% paraformaldehyde. After washing with PBS, fixed nerves were teased and transferred to glass slides,

permeabilized with 2% Triton X-100 in PBS for 30 minutes, and blocked in 10% goat serum and 1% Triton X-100 in PBS for 60 minutes at room temperature (RT). Then, double immunostaining was performed using mouse anti-Kv1.2 monoclonal antibodies (diluted 1:500) (NeuroMab, Davis, CA) and serum samples (diluted 1:100). Alexa 488-labeled antihuman IgG and Alexa 594-labeled antimouse IgG were used as secondary antibodies (Thermo Fisher Scientific) at a 1:500 dilution. The tissue-based IFAs were blindly assessed by 2 examiners (X.Z. and M.M.). In seropositive patients, IgG subclass profiles were examined using secondary fluorescein isothiocyanate-conjugated mouse monoclonal antihuman IgG1, IgG2, IgG3, and IgG4 antibodies (Sigma-Aldrich, St Louis, MO). Images were captured using a confocal laser scanning microscope (A1; Nikon, Tokyo, Japan) or BZ-X800 fluorescence microscope (KEYENCE, Osaka, Japan). The colocalization rate was obtained using the EzColocalization plugin from Image J (NIH, Bethesda, MD).

Cell-Based IFAs

Human embryonic kidney 293T cells maintained in a medium containing 10% fetal calf serum were seeded onto 8-well chamber slides (5×10^4 cells/well) 24 hours before transfection. The cells were transfected with both an LGI4-Flag plasmid (ID RC219293, OriGene Technologies, Inc., Rockville, MD) and a disintegrin and metalloprotease domain–containing protein 22 (ADAM22)–HA plasmid (constructed by M.F. and Y.F.)[16] or only an ADAM22-HA plasmid using lipofectamine 3000 transfection reagent (Thermo Fisher Scientific). At 24 hours after transfection, the cells were fixed with 2% paraformaldehyde at RT for 20 minutes and blocked with 20 mg/mL bovine serum albumin for 60 minutes on ice. Then, cells were incubated with the patients' serum IgG (diluted 1:200) or anti-LGI4 antibody (diluted 1:50) (Novus Biologicals) and anti-Flag antibody (diluted 1:1,000) (Sigma-Aldrich) at RT for 1 hour. After staining surface-bound human IgG, the cells were permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with PBS containing 20 mg/mL bovine serum albumin, and incubated with anti-HA polyclonal antibodies (Sigma-Aldrich), followed by staining with Alexa Fluor 488-conjugated or 594-conjugated secondary antibodies (Thermo Fisher Scientific). Images were captured using a confocal laser scanning microscope (A1, Nikon) or BZ-X800 fluorescence microscope (KEYENCE).

Extraction of Proteins From Tissue and Cell Lines

Lumbar DRG and sciatic nerves from mice and a Schwann cell line (IFRS1, COSMO BIO Co., Ltd, Japan) and human melanoma cell line (WM115, Rockland Immunochemicals, Limerick, PA) were homogenized in radioimmunoprecipitation lysis and extraction buffer (Nacalai Tesque, Kyoto, Japan) and centrifuged at 14,000 *g* for 10 minutes at 4°C. The supernatant fractions were collected and stored at −80°C.

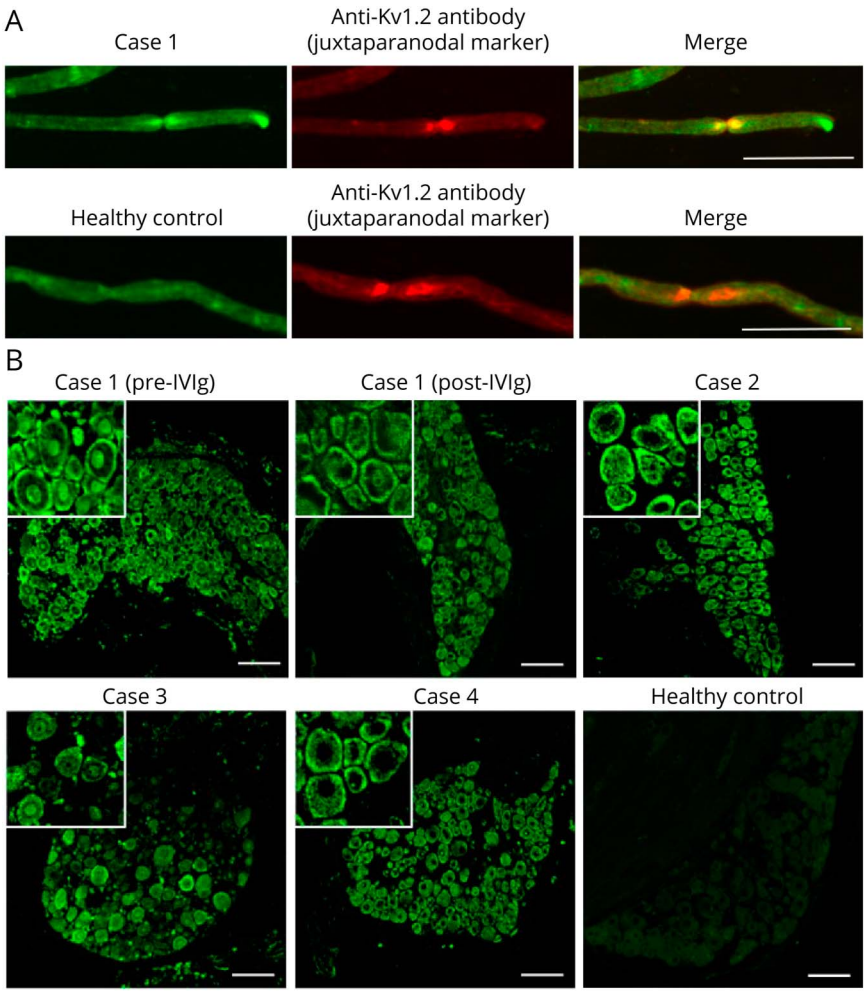
Western Blotting

Extracted proteins, LGI4 overexpression lysate (Novus Biologicals), which was generated in HEK293T cells using Plasmid ID RC219293 (OriGene Technologies, Inc.) based on accession number NM_139284, and other cell lysates were mixed with Laemmli sample buffer, heated at 95°C for 5 minutes, and then loaded onto polyacrylamide gradient gels. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Hertfordshire, UK). The membranes were then blocked in 3% skimmed milk for 1 hour and cut into strips. Each strip was separately incubated with sera from patients (1:300 dilution), rabbit anti-LGI4 antibody (1:1,000 dilution) (Abcam), or anti-Flag antibody (1:2,000 dilution, clone No. 1E6, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) overnight at 4°C. Membranes were washed and incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) for 1 hour at RT. Monoclonal anti-β–actin antibody (1:5,000 dilution) was used as an internal control and detected by horseradish peroxidase–conjugated secondary antibodies. Signals were detected using a ChemiDoc XRS system (Bio-Rad, Hercules, CA) or WSE-6270 LuminoGraph II EM (ATTO CORPORATION, Tokyo, Japan).

Cell-Based RNA Interference Assay

Complexes of Lipofectamine RNAiMAX (Thermo Fisher Scientific) and Silencer Select siRNA targeting *Lgi4* (siRNA ID: s167537) (Thermo Fisher Scientific) or scrambled siRNA negative control (#4390843) (Thermo Fisher Scientific) were added to rat Schwann cells. Transfection efficiency was assessed by quantitative real-time PCR and Western blotting (WB). The primer sequences are summarized in eTable 2, links.lww.com/NXI/A792. After confirming effective knockdown, cell-based IFAs were conducted using Schwann cells transfected with *Lgi4* siRNA or scrambled siRNA. Ninety-six hours after transfection, Schwann cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at RT, washed with PBS, and postfixed with 100% methanol for 10 minutes at −20°C. After blocking, the cells were incubated with sera from patients and controls (1:60 dilution) and rabbit polyclonal anti-LGI4 antibody (Novus Biologicals, 1:100 dilution) for 1 hour at RT and visualized using Alexa 488-conjugated antibodies to human IgG and Alexa 594-conjugated antibodies to rabbit IgG (Thermo Fisher Scientific, 1:1,000 dilution). Images were captured using a BZ-X800 fluorescence microscope (KEYENCE). The mean fluorescence intensity (MFI) was quantified using ImageJ (NIH). The same procedures were applied to the human melanoma cell line, which constitutively expresses LGI4, using Silencer Select siRNA targeting *LGI4* (siRNA ID: s46433) (Thermo Fisher Scientific) or scrambled siRNA negative control (#4390843) (Thermo Fisher Scientific). Total RNA was extracted from the siRNA-treated rat Schwann cells or human melanoma cells using a SuperPrepcCell Lysis & RT kit for quantitative PCR (#SCQ-101; TOYOBO, Osaka, Japan). The RNA was reverse transcribed using Fast SYBR Green Master Mix (Thermo Fisher Scientific) and subjected to quantitative real-time PCR analysis in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Genes of interest were compared and expressed as ratios relative to the reference gene, *glyceraldehyde-3-phosphate dehydrogenase* (Thermo Fisher Scientific).

Figure 1 Screening for Autoantibodies in Anti-neurofascin155 and Anti-contactin1 Antibody-Negative Patients With CIDP Using Mouse Sciatic Nerves and DRG



(A) Double immunostaining of a mouse teased sciatic nerve with a patient's serum and anti-Kv1.2 antibody shows colocalization of the patient's IgG with Kv1.2, a marker of juxtaparanodes. (B) IgG from 4 patients with CIDP, but not from a healthy control, demonstrates a circle-like staining pattern around the ganglion cells (see the magnified images). For case 1, immunostaining of mouse DRG by serum samples obtained before and 16 years after the first IVIg treatment is shown. Scale bar: 50 μm. Kv = potassium channel; CIDP = chronic inflammatory demyelinating polyneuropathy; DRG = dorsal root ganglia; IFA = immunofluorescence assay; IVIg = IV immunoglobulin.

Immunoadsorption Assay

In the immunoadsorption assay,[14] LGI4-Flag–cotransfected or nontransfected and ADAM22-HA–cotransfected or nontransfected HEK293T cells were grown in 6-well plates and incubated with a serum sample from a representative seropositive patient. The diluted serum (1:60 dilution) was used as the primary antibody and sequentially incubated in wells with transfected and nontransfected cells in parallel for 1 hour at 37°C and with 5% CO_2. After the reaction, we performed IFAs on mouse DRG as described earlier.

Evaluation of the Effects of Sera From Patients and Controls on Schwann Cells

Serum IgG from 3 novel nodal autoantibody-positive patients with CIDP (cases 1, 2, and 4), 3 seronegative patients with CIDP, and healthy participants were heated to 56°C for 30 minutes to inactivate any potential complement factors, diluted 1:10 in cell culture medium, and added to plated rat Schwann cells, which were then incubated overnight. Thereafter, quantitative real-time PCR was conducted to compare *Krox20* and *Periaxin* expression among the seropositive and seronegative patients with CIDP and healthy controls. Cell-based IFAs were conducted using rat Schwann cells and anti-ADAM22 polyclonal antibody (Abcam) or anti-LGI4 antibody (diluted 1:100) (Novus Biologicals). Both quantitative real-time PCR and cell-based IFAs followed the methods mentioned earlier.

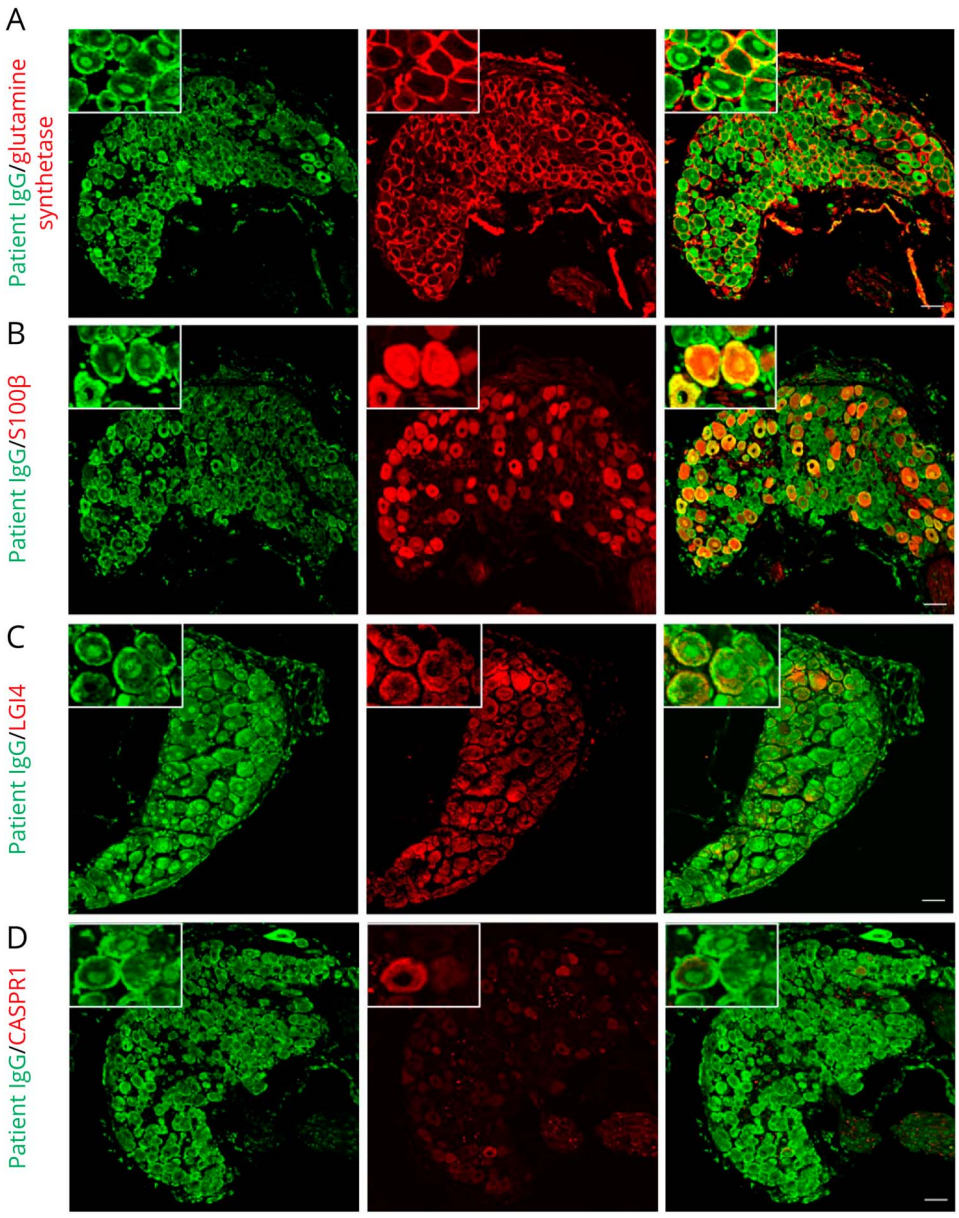
Standard Protocol Approvals, Registrations, and Patient Consents

The research protocol for this retrospective study and the data privacy procedures for human samples were approved by the Ethics Committees of the International University of Health and Welfare (20-Ifh-025) and Kyushu University (2020-789). An opt-out recruitment method was adopted. This study was conducted according to the Strengthening the Reporting of Observational Studies in Epidemiology reporting guidelines.[17]

Statistical Analysis

All analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). Paired and unpaired *t* tests were used for comparisons. Statistical significance was set at $p < 0.05$.

Figure 2 Identification of Anti-LGI4 Antibodies in Sera From Tissue-Based IFA-Positive Patients With CIDP by DRG-Based IFAs



Double immunostaining of mouse DRG with serum from a tissue-based IFA-positive patient with CIDP (case 1 in Table 1) and different commercial antibodies. (A) Immunostaining of glutamine synthetase (red), a specific satellite glial cell marker, colocalized with patient IgG binding (green). (B) Immunostaining of S100β (red), a marker of large DRG neurons and satellite glial cells, partly colocalized with patient IgG binding (green). (C) IgG binding of the patient's serum (green) colocalized with that of the commercial anti-LGI4 antibody (LGI4; red). (D) immunostaining of CASPR1 (red), a paranode marker, which mainly stained sensory neuron somata, barely colocalized with patient IgG binding (green). Scale bar: 50 μm. CIDP = chronic inflammatory demyelinating polyneuropathy; CASPR1 = contactin-associated protein 1; DRG = dorsal root ganglia; IFA = immunofluorescence assay; LGI4 = leucine-rich repeat LGI family member 4.

Data Availability

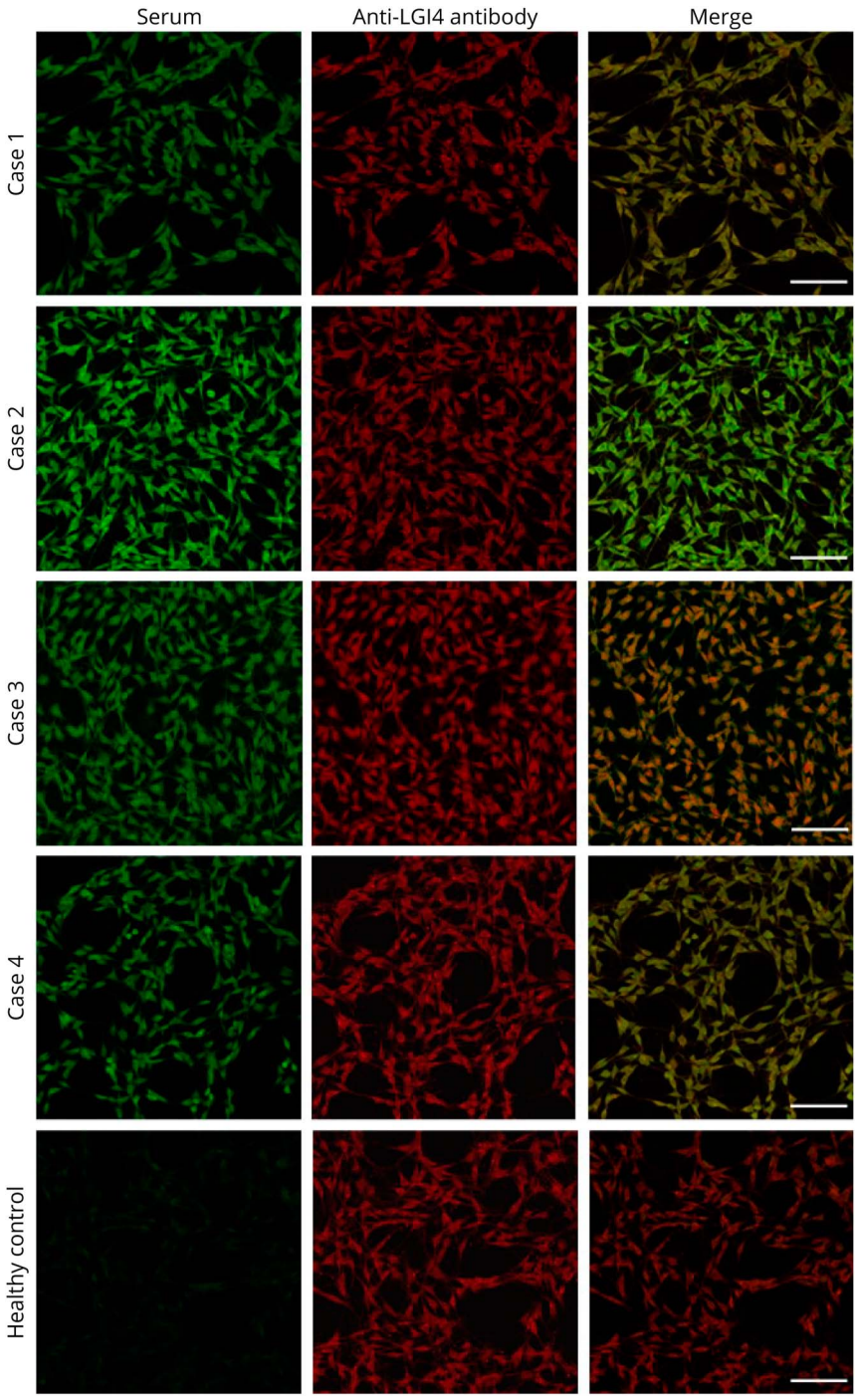
Any data not published within the article will be shared in anonymized form upon request from any qualified investigator.

Results

Serum Antibodies From Some Anti-NF155 and CNTN-1 Antibody-Seronegative CIDP Patients Bind to Teased Fibers of Sciatic Nerves and DRG

Among the 113 seronegative patients with CIDP, the age at examination was 52 ± 17 years (range 18–91 years), and the age at onset was 46 ± 18 years (range 5–86 years). The CIDP subtypes included typical in 68, distal acquired demyelinating symmetric in 18, multifocal acquired demyelinating sensory and motor neuropathy (MADSAM) in 11, pure sensory in 7, focal in 5, pure motor in 1, and undetermined in 3 patients. Serum samples from 4 of 113 anti-NF155 and CNTN-1 antibody–negative patients with CIDP bound to mouse sciatic nerve fibers and DRG in tissue-based IFAs (Figure 1, A and B). For sciatic nerve fibers, IgG from these 4 patients selectively reacted with the juxtaparanodal regions of nodes of Ranvier, which overlapped with reactivity with Kv1.2, a juxtaparanodal marker protein (Figure 1A). For DRG, IgG from all 4 patients showed a characteristic binding pattern with a

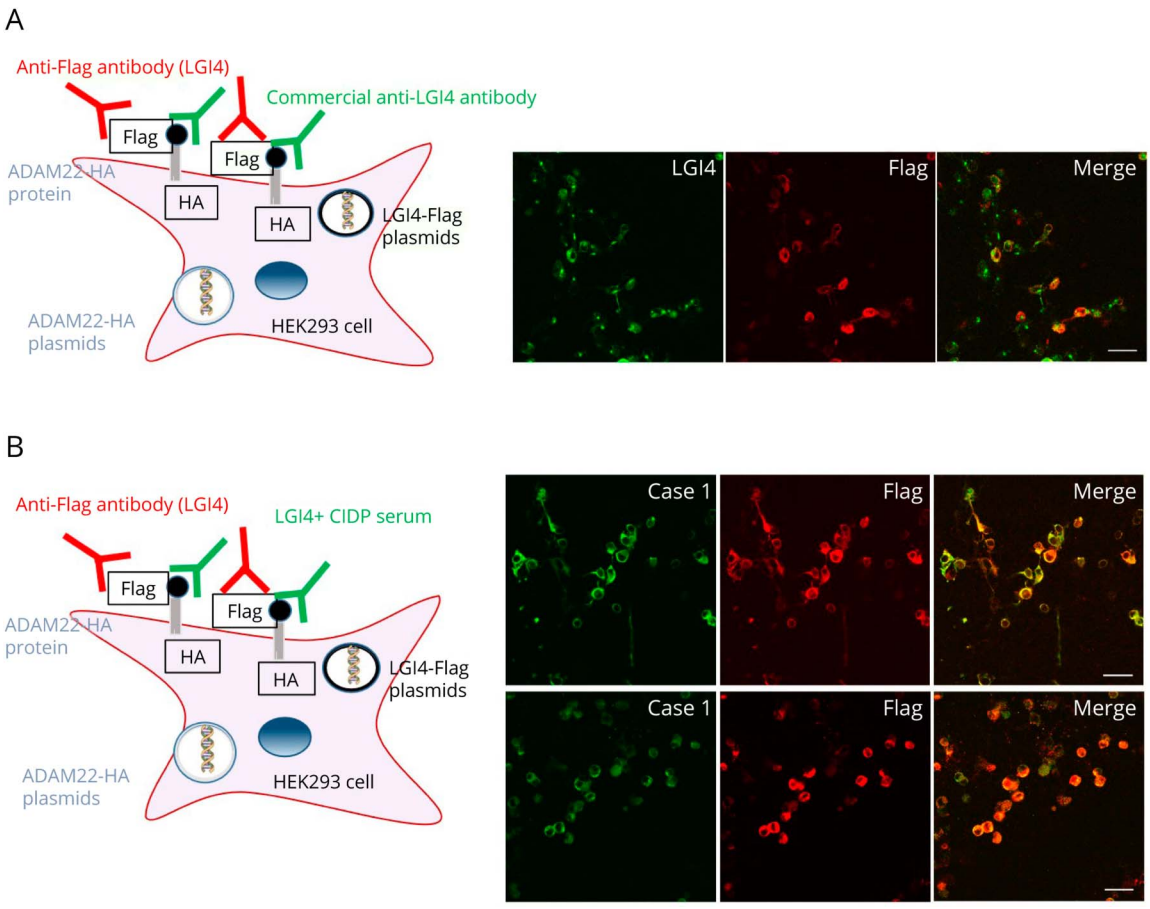
Figure 3 Cell-Based IFA Using Rat Schwann Cells With Patients' IgG and a Commercial Anti-LGI4 Antibody



Serum samples from 4 seropositive patients with CIDP and a commercial anti-LGI4 antibody all demonstrate the same immunostaining pattern on rat Schwann cells, while a healthy control shows no immunoreactivity. Scale bars: 20 μm. CIDP = chronic inflammatory demyelinating polyneuropathy; IFA = immunofluorescence assay; LGI4 = leucine-rich repeat LGI family member 4.

circle-like appearance around ganglion cells (Figure 1B). The patients' IgG colocalized with glutamine synthetase (Figure 2A), a specific satellite glial cell marker.[18] Immunostaining of S100β, a myelinated Aβ-fiber–type and Aδ-fiber–type DRG neuron and satellite glial cell marker,[19] showed partial overlap with IgG binding in the patients (Figure 2B). These results indicated that patient IgG binding is confined to DRG satellite glia. One hundred twenty-seven control sera showed no apparent immunoreactivity to DRG (Figure 1B). Therefore, the positivity rate for anti-DRG satellite glial cell antibodies was higher in the CIDP group than that in the control group (4/113 [3.5%] vs 0/127 [0%], $p = 0.048$) (eTable 1, links.lww.com/NXI/A792). IgG4 was predominant in all 4 positive patients, but other IgG subclasses also coexisted in all cases (case 1: IgG1 and 2, case 2: IgG1, 2, and 3; cases 3 and 4: IgG2 and 3) (eFigure 1, links.lww.com/NXI/A792).

Figure 4 Cell-Based IFAs Using Commercial Anti-LGI4 Antibodies and Sera of Seropositive Patient With CIDP



(A) LGI4-Flag–cotransfected and ADAM22-HA–cotransfected HEK293T cells stained with anti-Flag and commercial anti-LGI4 antibodies. (B) LGI4-Flag–cotransfected and ADAM22-HA–cotransfected HEK293T cells stained with anti-Flag antibodies and IgG from seropositive patients with CIDP (cases 1 and 4) showed cell surface staining. Scale bar: 50 μm. ADAM22 = a disintegrin and metalloprotease domain–containing protein 22, CIDP = chronic inflammatory demyelinating polyneuropathy; HEK = human embryonic kidney; IFA = immunofluorescence assay; LGI4 = leucine-rich repeat LGI family member 4.

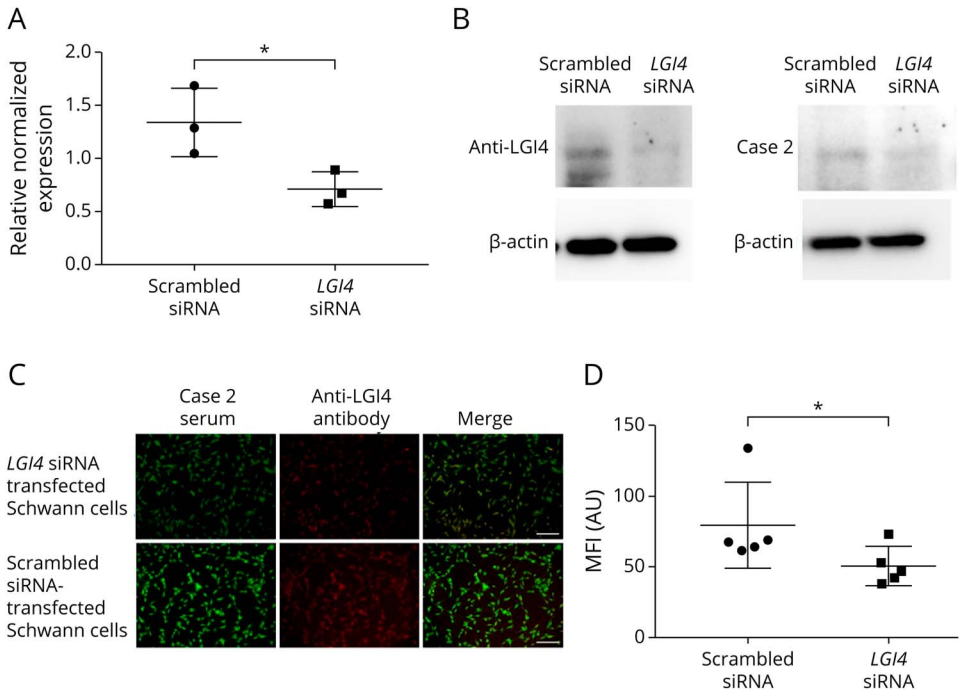
Immunochemical Characterization of Autoantigens

WB using proteins extracted from mouse DRG revealed that serum IgG from the tissue-based IFA-positive patients with CIDP commonly stained a band of approximately 60 kDa, while that from healthy controls did not (eFigure 2A, links.lww.com/NXI/A792). Immunostaining of cultured rat Schwann cells with the positive patients' sera also exhibited positive staining for all 4 cases but not for healthy controls (Figure 3). Therefore, we compared the proteins expressed in both Schwann cells[20] and DRG[21] and found 8 common genes encoding 55–65 kDa proteins, namely, *B3glct*, *Krt10*, *Lgi4*, *Tyrp1*, *Pgm1*, *Thbd*, *Gatb*, and *Plekha4*. Among these candidates, *Lgi4* is the only gene involved in peripheral nerve myelination.[22,23] LGI4 is mainly expressed in Schwann cells, enteric glia, and DRG satellite glia,[24] and its calculated molecular weight is 59,377 Da.[22] Thus, we considered LGI4 as a candidate antigen. A commercial anti-LGI4 antibody demonstrated an immunostaining pattern of mouse DRG in a tissue-based IFA similar to that of the positive patients' IgG (Figure 2C), presenting a colocalization rate according to the Spearman rank correlation coefficient of 0.52 ± 0.15, indicating a moderately positive relationship between the 2 signals. Furthermore, these markers showed a median Manders' colocalization coefficient for M1 (fraction of LGI4-containing patient IgG) of 0.857, and that for M2 (fraction of patient IgG containing LGI4) was 0.841, indicating a moderate to high level of colocalization in both directions. However, CASPR1, a paranode marker,[7] which only weakly stained sensory neuron somata, barely colocalized with patient IgG binding (Figure 2D). Furthermore, IgG from all 4 patients with CIDP bound to the 60-kDa protein in Western blots of LGI4 overexpression cell lysates (eFigure 2B, links.lww.com/NXI/A792).

Verification of Autoantibody Specificity for LGI4 by Cell-Based IFAs and WB

A commercial anti-LGI4 antibody reacted with rat Schwann cells in IFAs, presenting the same immunostaining pattern as the sera of 4 seropositive patients with CIDP (Figure 3). Sera

Figure 5 Confirmation of the Autoantibody Specificity for LGI4 Using a Genetic Strategy With Rat Schwann Cells



(A) Assessment of the effect of *Lgi4* siRNA transfection on *Lgi4* mRNA levels in rat Schwann cells by quantitative real-time PCR. The results are expressed as mean ± SEM. The expression of *glyceraldehyde-3-phosphate dehydrogenase* was determined as a housekeeping gene. *p = 0.0213. (B) WB analysis of LGI4 protein using rat Schwann cells after *Lgi4* siRNA or scrambled siRNA treatment. IgG from 1 representative seropositive patient with CIDP (case 2) and a commercial anti-LGI4 antibody showed decreased signals after *Lgi4* siRNA treatment compared with the scrambled siRNA in rat Schwann cells. (C) Cell-based IFA using *Lgi4* siRNA or scrambled siRNA-treated rat Schwann cells. Signals in rat Schwann cells from the serum of case 2 and the anti-LGI4 antibody were significantly decreased after *Lgi4* siRNA treatment compared with those after scrambled siRNA treatment. (D) Comparison of the MFI from a CIDP patient's IgG between *Lgi4* siRNA and scrambled siRNA-treated rat Schwann cells. Nuclei are counterstained with DAPI (blue). The results are expressed as the mean ± SEM. *p = 0.0241. Scale bars: (C) 30 μm. AU = arbitrary units; CIDP = chronic inflammatory demyelinating polyneuropathy; DAPI = 4′,6-diamidino-2-phenylindole; IFA = immunofluorescence assay; LGI4 = leucine-rich repeat LGI family member 4; MFI = mean fluorescence intensity; WB = Western blotting.

from all 4 patients with CIDP, but not healthy controls, and the commercial anti-LGI4 antibody recognized a 60-kDa protein band in Western blots of rat Schwann cell lysates (eFigure 2C, links.lww.com/NXI/A792).

To express the secretory protein LGI4 on the cell surface, LGI4-Flag plasmids and ADAM22-HA plasmids (HA was added inside the cells) were cotransfected into HEK293T cells. The secreted Flag-tagged LGI4 was immunostained with anti-Flag antibodies and was present on the cell surface near HA-tagged ADAM22 immunostained with anti-HA antibodies (eFigure 3A, links.lww.com/NXI/A792), which suggests specific interactions between LGI4 and ADAM22. The commercial anti-LGI4 antibody bound to the cotransfected cells but not naive HEK293T cells (Figure 4A and eFigure 3B, links.lww.com/NXI/A792), which further confirmed transfected LGI4 expression. Serum IgG from 3 seropositive patients with CIDP, except for case 3, whose serum was unavailable, reacted with the LGI4-Flag–cotransfected cells and ADAM22-HA–cotransfected cells but not with ADAM22-HA–transfected cells (Figure 4B and eFigure 4A and B, links.lww.com/NXI/A792). None of the 127 control sera samples reacted with the LGI4-Flag–cotransfected cells and ADAM22-HA–cotransfected cells (eFigure 4C, links.lww.com/NXI/A792).

Confirmation of Autoantibody Specificity for LGI4 by a Genetic Strategy and Immunoadsorption

IgG from a representative seropositive patient (case 2) was used in a genetic strategy. In a cell-based RNA interference assay, *Lgi4* siRNA treatment of rat Schwann cells induced a substantial decrease in *Lgi4* mRNA expression (p = 0.0213, n = 3 cultures/group) and LGI4 protein levels compared with scrambled siRNA treatment, as assessed by quantitative real-time PCR and WB, respectively (Figure 5, A and B). In accordance with this decrease, the WB and cell-based IFAs showed that both IgG from the representative seropositive patient (case 2) and the commercial anti-LGI4 antibody produced significantly decreased signals after *Lgi4* siRNA treatment compared with the scrambled siRNA treatment (Figure 5, B and C). *Lgi4* siRNA treatment decreased the MFI of IgG binding signals of the representative patient with CIDP (case 2) by 63% compared with those detected after scrambled siRNA treatment (p = 0.0241, n = 5 cultures/group) in rat Schwann cells (Figure 5D). Similar results were obtained in cell-based interference assays when we used a human melanoma cell line that constitutively expresses LGI4 (eFigure 5, links.lww.com/NXI/A792). In addition, the immunoreactivity of serum IgG from a representative anti-LGI4 antibody–positive patient (case 1 in Table 1) was markedly reduced by preincubation with LGI4-Flag–cotransfected

Table 1 Clinical Features of Patients With CIDP With Anti-LGI4 Antibodies

	Case 1	Case 2	Case 3	Case 4
Age, y/sex	42/female	74/male	76/female	51/male
Age at onset, y	42	64	76	51
Clinical phenotype	Typical	MADSAM	Typical	Typical
Mode of onset	Subacute[a]	Chronic	Subacute[a]	Subacute[a]
Symptoms and signs				
Initial symptoms	Motor & sensory	Motor & sensory	Motor & sensory	Motor & sensory
Limb weakness	+	+	+	+
Muscle atrophy	+	+	−	−
Disturbance of superficial sensation	+	+	+	+
Disturbance of deep sensation	+	+	+	+
Glove and stocking type sensory disturbance	+	−	+	+
Romberg sign	Unable to examine	+	+	+
Ataxia	Unable to examine	+	+	−
Tremor	+ (finger)	+ (finger)	−	−
CSF tests				
CSF protein amounts (mg/dL)	363	182	253	541
CSF cell counts (/μL)	1	1	0	15
MRI				
Spinal root hypertrophy	−	+	−	−
Treatment[b]				
Oral steroids	Not performed	Not performed	Partially effective	Ineffective
IV Steroids	Not performed	Not performed	Not available	Not performed
IVIg	Partially effective	Partially effective	For maintenance therapy following the steroid therapy	Partially effective
Others	Not performed	Not performed	Not performed	Not performed

Abbreviations: CIDP = chronic inflammatory demyelinating polyneuropathy; IVIg = IV immunoglobulin; LGI4 = leucine-rich repeat LGI family member 4; MADSAM = multifocal acquired demyelinating sensory and motor neuropathy.
Romberg sign and ataxia were not examined because of severe limb weakness in case 1.
[a] Subacute onset was defined as taking 2–8 weeks to reach the peak deficit after the initial attack in this study (7 week in case 1, 2 weeks in case 3, and 6 weeks in case 4).
[b] Evaluation of motor impairment before and 2–4 weeks after immunotherapy: IVIg in case 1; hand grasping power (right/left), 3/3–5/4 kg, and straight leg raising test, 0°–90° on both sides. IVIg in case 2: hand grasping power (right/left), 13/10–25/13 kg. Methylprednisolone pulse therapy followed by oral prednisolone (55 mg/d) with gradual taper in case 3: proximal lower limb muscle power, 2/5–3/5 by manual muscle testing on both sides. IVIg was used only for maintenance therapy, and no recurrence was observed during the follow-up period in case 3. IVIg in case 4: from inability to stand to walking with a walker and extension and flexion of finger and knee joints (muscle power), 2/5–3/5 on both sides.

HEK293T cells and ADAM22-HA–cotransfected HEK293T cells but not by preincubation with nontransfected HEK293T cells (eFigure 6, links.lww.com/NXI/A792).

Effects of Sera From Patients and Controls on Schwann Cells

To assess the effects of patients' anti-LGI4 antibodies on Schwann cells, we examined whether anti-LGI4 antibody-positive ($LGI4^+$) CIDP patient serum affects the expression of *Krox20* and *Periaxin*, both of which independently control Schwann cell myelination.[25,26] First, a cell-based IFA confirmed that ADAM22, an LGI4 receptor,[22,24] is expressed by these Schwann cells (eFigure 7A, links.lww.com/NXI/A792). Second, treatment of Schwann cells with serum IgG from either $LGI4^+$ patients with CIDP, seronegative patients with CIDP, or healthy controls did not apparently alter LGI4 protein expression in Schwann cells (eFigure 7B, links.lww.com/NXI/A792). Third, *Krox20* mRNA levels were decreased in Schwann cells treated with serum IgG from $LGI4^+$ patients with CIDP compared with those of Schwann cells

treated with serum IgG from seronegative patients with CIDP ($p = 0.0045$) and healthy controls ($p = 0.03$) (n = 3 patients/group) (Figure 6A). No significant changes in *Periaxin* mRNA levels were observed after treatment (Figure 6B).

Clinical Features of Patients With CIDP With Anti-LGI4 Antibodies

The clinical findings of the 4 LGI4$^+$ patients with CIDP are summarized in Table 1. These patients had a relatively old age at onset (mean age: 58 years, range 42–76). Three presented with typical CIDP and 1 presented with MADSAM. The mode of onset was subacute in all 3 typical cases and chronic in the MADSAM case. All 4 patients had deep sensory impairment showing positive Romberg sign in addition to motor weakness. The 3 typical cases showed a glove and stocking type sensory disturbance. Finger tremor was seen in 2 patients. All patients had extremely high CSF protein levels (mean 335 mg/dL, range 182–541), while spinal root hypertrophy was detected in only 1 patient with the longest disease duration (10 years). IVIg was partially effective in the 3 patients to whom it was administered for remission-induction therapy, although a circle-like staining pattern around the ganglion cells with case 1 serum IgG was still obvious even after IVIg treatment (Figure 1B).

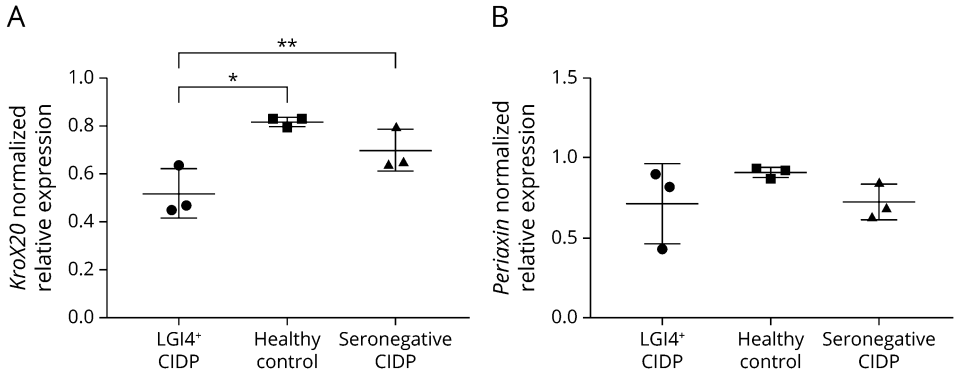
Discussion

In this study, we found 4 anti-NF155 and anti-CNTN1 antibody-seronegative patients with CIDP who harbored IgG4 antibodies against the paranodal regions of peripheral myelinated fibers and DRG satellite glia. The reactivity of the patients' IgG to ADAM22-cotransfected cells and LGI4-cotransfected cells and the reduction in the patients' IgG staining of rat Schwann cells constitutively expressing LGI4 by *LGI4* siRNA treatment and preincubation with ADAM22-cotransfected cells and LGI4-cotransfected cells convincingly indicated the autoantibody specificity for LGI4. Because patient IgG positively reacted to LGI4 in tissue-based and cell-based IFAs and WB assays, as observed for other antineural autoantibodies,[5,14] autoantibodies may recognize both conformational and linear epitopes.

The 4 LGI4$^+$ patients with CIDP exhibited unique features, such as severe proprioceptive sensory impairment causing positive Romberg's sign and ataxia in addition to motor weakness, and extremely high CSF protein levels. In addition, 2 patients had finger tremor. These features are common in NF155$^+$ and CNTN1$^+$ CIDP.[3-7,9-12,27] As for the relatively old age at onset, LGI4$^+$ patients with CIDP were similar to CNTN1$^+$ patients with CIDP.[11-14] Notably, all 3 typical patients with CIDP showed subacute onset, mimicking Guillain-Barré syndrome, as seen for other nodal autoantibodies.[3,6,10-12] Spinal root hypertrophy was detected only in 1 patient with MADSAM, who had the longest disease duration of 10 years. In NF155$^+$ CIDP, root hypertrophy is associated with longer disease duration.[8] Because all LGI4$^+$ typical patients with CIDP had subacute onset and short disease duration, root hypertrophy may not have emerged.

LGI4 belongs to the LGI family of proteins, which plays important roles in cell-cell interactions.[22,28] Among LGI family members, LGI1 is a relevant antigen for autoimmune limbic encephalitis.[29-31] LGI1 interacts with ADAM22 and ADAM23 at synaptic clefts of hippocampal neurons.[29-31] IgG4 anti-LGI1 antibodies disrupt interaction between LGI1 and ADAM22.[31] However, LGI1 does not seem to be expressed in DRG neurons in culture.[32] In peripheral nerves, LGI4 secreted from Schwann cells induces myelination through a paracrine mechanism through ADAM22 expressed on the axonal membrane, which is indispensable for motor nerve myelination, while the autocrine mechanism of Krox20-positive Schwann cells is important for sensory nerve myelination.[22,33,34] Ablation of either LGI4 or ADAM22 in mice induces peripheral nerve hypomyelination.[24,34] In humans, loss-of-function *LGI4* variations cause neurogenic arthrogryposis multiplex congenita with peripheral myelin defects.[23,35,36] Given the reduction of *Krox20* mRNA levels in Schwann cells by LGI4$^+$ CIDP patient serum, both paracrine and autocrine processes may be impaired by IgG4 anti-LGI4 antibodies. LGI4 is also expressed in DRG satellite glia, and

Figure 6 Effects of Sera From Anti-LGI4 Antibody-Seropositive Patients With CIDP and Controls on Schwann Cells



Quantitative real-time PCR assessment of *Krox20* (A) and *Periaxin* (B) expression in rat Schwann cells. The results are expressed as the mean ± SEM. *p = 0.03, **p = 0.0045. CIDP = chronic inflammatory demyelinating polyneuropathy; LGI4 = leucine-rich repeat LGI family member 4.

secreted LGI4 induces their proliferation through binding to ADAM22.[24] Therefore, anti-LGI4 antibodies may invade DRG where the blood-nerve barrier is absent and disrupt interaction between satellite glia and DRG neurons. Such a mechanism may contribute to the severe proprioceptive sensory impairment in LGI4$^+$ CIDP.

IgG4 cannot activate the complement cascade or internalize the target antigens,[37] which may explain the lack of apparent alteration of surface LGI4 when patient IgG was applied to cultured Schwann cells. Thus, IgG4 anti-LGI4 antibodies may block critical interaction between LGI4 and ADMA22 or between LGI4s because dimerization between LGI1s and LGI1 is required for proper functioning in the case of LGI1-ADAM22 complexes, and the LGI1-LGI1 interaction sites are also conserved in other LGIs including LGI4.[38]

IgG4-mediated neurologic autoimmunity usually shows a poor response to IVIg.[37] However, in our series, partial responses to IVIg treatment were observed in 3 LGI4$^+$ patients with CIDP, who also had IgG1–3 subclass autoantibodies in addition to the dominant IgG4 subclass. Inhibition of coexisting IgG1–3 autoantibody functions by IVIg may explain this partial response.[37] In addition, case 3 partially responded to corticosteroids, which is consistent with the reported favorable response to corticosteroids of LGI1-associated autoimmune limbic encephalitis.[39] Thus, corticosteroids may also be an alternative choice in this condition.

Our study had several limitations. First, we did not measure anti-CASPR1 or antipan-neurofascin antibodies. However, double IFAs using mouse DRG with patient IgG and a commercial anti-CASPR1 antibody revealed that the anti-CASPR1 antibody weakly stained sensory neuron somata, which sparsely colocalized with patient IgG binding. This anti-CASPR1 antibody staining pattern is consistent with previous reports.[40,41] In addition, none of the LGI4$^+$ patients with CIDP had anti-NF155 antibodies. If these patients harbored antipan-neurofascin antibodies, they should have had positive results on the anti-NF155 antibody assay. Therefore, we considered it unlikely that our patients carried anti-CASPR1 or antipan-neurofascin antibodies. Second, case 3 serum was not available for the cell-based assay using HEK293T cells cotransfected with ADAM22 and LGI4 expression plasmids; however, other study results including tissue-based IFAs using DRG, sciatic nerves, and cultured Schwann cells and WB showed that case 3 serum reactivity was essentially the same as that of the other 3 cases, suggesting anti-LGI4 antibodies were present. Finally, although the in vitro Schwann cell culture study indicated some biological effects of serum of LGI4$^+$ patient with CIDP patient, in vivo animal studies that reproduce peripheral nerve demyelination with patient serum remain to be conducted to verify the pathogenicity of anti-LGI4 antibodies.

In conclusion, a fraction of patients with CIDP harbor IgG4 antibodies against LGI4 and show severe sensory impairment in addition to motor weakness. Thus, anti-LGI4 antibodies should be examined in autoimmune nodopathy–type patients with CIDP, particularly in subacute onset cases, if they are negative for anti-NF155 and anti-CNTN1 antibodies. Further exploration of anti-LGI4 antibodies in patients with CIDP is warranted to elucidate the full spectrum of IgG4 autoantibody-mediated autoimmune nodopathy to improve clinical diagnosis and develop optimal treatment.

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