Neurofascin IgG4 antibodies in CIDP associate with disabling tremor and poor response to IVIg

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ABSTRACT

Objective: To describe the frequency of antibodies against neurofascin in chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and the associated clinical features.

Methods: Immunocytochemistry was used to identify antibodies to neurofascin 155 (NF155) and 186. Serum reactivity with paranodes and brain tissue was tested with immunohistochemistry of teased-nerve fibers and rat brain. Antibody titers and immunoglobulin (Ig) G isotypes were determined using ELISA. Clinical information was obtained retrospectively.

Results: Two of 53 patients, but none of 204 controls, had antibodies to NF155 ($p = 0.041$). The 2 patients with NF155 antibodies developed severe polyradiculoneuropathy with predominant distal weakness that was refractory to IVIg. Eight additional patients with IVIg-refractory CIDP were then identified from a national database; 2 of them with the same clinical features also had NF155 antibodies. Overall, 3 of the 4 patients with NF155 antibodies had a disabling and characteristic tremor (high amplitude, low frequency, postural, and intention). Patients' antibodies reacted with the paranodes in teased-nerve fibers and with the neuropil of rat cerebellum, brain, and brainstem. Anti-NF155 antibodies were predominantly of the IgG4 isotype in all patients.

Conclusion: Patients with CIDP positive for IgG4 NF155 antibodies constitute a specific subgroup with a severe phenotype, poor response to IVIg, and disabling tremor. Autoantibodies against paranodal structures associate with distinct clinical features in CIDP and their identification has diagnostic, prognostic, and therapeutic implications.

Classification of evidence: This study provides Class IV evidence that autoantibodies to NF155 identify a CIDP subtype characterized by severe neuropathy, poor response to IVIg, and disabling tremor. *Neurology*® 2014;82:879–886

GLOSSARY

CCPD = combined central and peripheral demyelination; **CIDP** = chronic inflammatory demyelinating polyradiculoneuropathy; **CMAP** = compound motor action potential; **CNTN1** = contactin-1; **GBS** = Guillain-Barré syndrome; **Ig** = immunoglobulin; **IVIg** = IV immunoglobulin; **MS** = multiple sclerosis; **NF155** = neurofascin 155; **NF186** = neurofascin 186; **NFASC** = neurofascin; **PBS** = phosphate-buffered saline; **PEx** = plasma exchange; **RT** = room temperature.

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a heterogeneous polyneuropathy of autoimmune origin.[1] Diagnosis relies on clinical and neurophysiologic criteria[2]; useful diagnostic biomarkers are lacking.[3] A good response to IV immunoglobulin (IVIg) and plasma exchange (PEx)[4,5] and experimental data[6–8] support a role of autoantibodies in CIDP pathogenesis. Recent pathologic studies demonstrate the disruption of nodes of Ranvier in peripheral nerves of patients with CIDP, suggesting that the node of Ranvier or related structures could be the target of the immune response.[9–11] Several groups have described autoantibodies against nodal and paranodal proteins such as neurofascin (NFASC), NrCAM, and gliomedin in CIDP.[12–15] We recently described a small subset of patients with CIDP with anti–contactin-1 (CNTN1) antibodies who share aggressive onset and poor response to IVIg, suggesting that the presence of

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specific autoantibodies may define clinical phenotypes.[16] However, the clinical features of CIDP associated with other autoantibodies have not been precisely described.

NFASC is present in node of Ranvier structures as 2 isoforms. Neurofascin 186 (NF186) is located in the axonal membrane and is essential for sodium-channel clustering at the node of Ranvier.[17,18] Neurofascin 155 (NF155) is located in the paranodal loops of myelinating Schwann cells where it is necessary to form septate-like axo-glial junctions.[19] Autoantibodies against both NFASC isoforms have been described in CIDP,[12,13] Guillain-Barré syndrome (GBS),[12–14] and multiple sclerosis (MS),[20] and patients with combined central and peripheral demyelination (CCPD) have an increased frequency of anti-NF155 antibodies.[21] The aim of this study was to describe the clinical features of patients with CIDP and antibodies against NFASC.

METHODS

Patients and samples. Sera from 53 patients meeting the European Federation of Neurological Societies/Peripheral Nerve Society task force CIDP diagnostic criteria[2] followed in the Neuromuscular Disorders Unit at Hospital de la Santa Creu i Sant Pau were initially studied. Sera and clinical information were obtained from an additional cohort of 8 IVIg-resistant patients from a Spanish National Registry (CIBERNED-CIDP) and have been included in a previously published cohort.[22] Control samples included 204 patients with other neuromuscular disorders, including GBS and other immune neuropathies (table e-1 on the *Neurology*® Web site at Neurology.org).

Immunocytochemistry. HEK293 cells were plated on poly-D-lysine–coated coverslips in fetal bovine serum–supplemented Dulbecco modified Eagle culture medium for 24 hours. The pIGplus expression vector containing cDNA encoding the extracellular domain of rat NF155 (courtesy of Catherine Faivre-Sarrailh, University of Marseille, and Dr. Peter Brophy, University of Edinburgh)[23] and an expression vector encoding for rat hemagglutinin-tagged NF186 cDNA (courtesy of Jerome Devaux, University of Marseille, and Dr. Bennett, Duke University)[12] were transfected into HEK293 cells at 70% to 80% confluence using lipofectamine 2000 (Life Technologies, Madrid, Spain). After 24 hours, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes, washed, and blocked 1 hour with 5% normal goat serum in PBS. Coverslips were frozen until needed.

Sera (1:100 concentration) and chicken polyclonal anti-NF155 (R&D Systems, Minneapolis, MN) were diluted in blocking buffer and incubated with coverslips for 1 hour at room temperature (RT). Coverslips were then washed and incubated with mouse anti-human targeting the immunoglobulin (Ig)G light chains κ/λ (Vector Labs, Burlingame, CA). Alexa-Fluor goat anti-human (594) IgG and IgM and anti-chicken (488) secondary antibodies (Molecular Probes, Eugene, OR) were incubated with coverslips 1 hour at RT, washed, and mounted onto slides with Vectashield with DAPI (diamidino-2-phenylindole) (Vector Labs).

Teased-nerve immunohistochemistry. Adult Lewis rats were sacrificed by CO_2 inhalation, and the sciatic nerves were removed. Teased-nerve fibers were transferred to slides in PBS, dried at RT, and frozen at −20°C until use. Slides containing teased-nerve fibers were thawed and blocked with 5% normal goat serum in PBS for 1 hour. Anti-NF155–positive sera (1:50 concentration) and chicken polyclonal anti-NF155 were incubated with teased fibers. Appropriate Alexa-Fluor goat anti-human (594) and anti-chicken (488) were used as secondary antibodies. Slides were mounted with Fluoprep (bioMérieux, Marcy l'Etoile, France).

Rat brain immunohistochemistry. Nonperfused rat brains were removed, split sagittally, and fixed in 4% paraformaldehyde for 1 hour, cryoprotected with 40% sucrose for 24 hours, and snap frozen in chilled isopentane. Seven-micron-thick sections were then incubated with 0.3% hydrogen peroxide for 20 minutes, 10% goat serum in PBS for 1 hour, and then with patient or control serum (dilution 1:200) at 4°C overnight. The next day, sections were washed and incubated with biotinylated goat anti-human IgG diluted 1:2,000 (Vector Labs) for 2 hours at RT, and the reactivity was visualized with an avidin-biotin-peroxidase method.[24]

Immunoabsorption. Nontransfected and NF155-transfected HEK293 cells were grown in 6-well plates, fixed with 4% paraformaldehyde in PBS, and incubated with anti-NF155+ CIDP sera (1:40) in 5% goat serum in PBS 1 hour per well. Supernatants from both transfected and nontransfected plates were used for rat teased-nerve immunohistochemistry experiments.

ELISA. Ninety-six-well Nunc MaxiSorp ELISA plates (Fisher Scientific, Madrid, Spain) were coated at 4°C with 1 μg/mL human recombinant NF155 protein (OriGene, Rockville, MD) overnight. Wells were blocked with 5% nonfat milk in PBS for 1 hour at RT and then incubated with sera diluted in blocking buffer for 1 hour at RT. Horseradish peroxidase–labeled rabbit anti-human IgG (Dako, Glostrup, Denmark) was then added for 1 hour at RT. To detect levels of the different IgG isotypes, appropriate horseradish peroxidase–labeled mouse anti-human IgG1–4 secondary antibodies were used (Invitrogen, Carlsbad, CA). ELISA was developed with TMB solution (BioLegend, San Diego, CA) and the reaction stopped with 1 M sulfuric acid. Optical density was measured at 450 nm in a Beckman AD340 plate reader (Beckman-Coulter, Indianapolis, IN). All samples were tested in duplicate. To calculate the anti-NF155 titers, the ELISA was performed with different serum concentrations (range 1:100–1:200,000) and the test was considered positive when the optical density was 2 SDs above the average healthy control ($n = 5$) signal. The last dilution testing positive was set as the anti-NF155 titer.

Standard protocol approvals and patient consents. Clinical data from patients followed in our unit were collected and recorded in a coded database. Patients' samples and written informed consent were obtained under a protocol approved by the Ethics Committee of the Hospital de la Santa Creu i Sant Pau. Patients from the CIBERNED-CIDP registry participated in a study published elsewhere,[22] in which a specific informed consent for their inclusion in the registry was obtained. All procedures using animals were approved by the Animal Experimentation Committee of the Institut de Recerca Hospital de Sant Pau, Barcelona, Spain. All patients provided informed consent to participate in the study. A specific informed consent was obtained for video recordings.

Statistical analyses were performed with GraphPad Prism version 5.0 (La Jolla, CA).

Classification of evidence. The primary objectives of our study were to describe the frequency of antibodies against

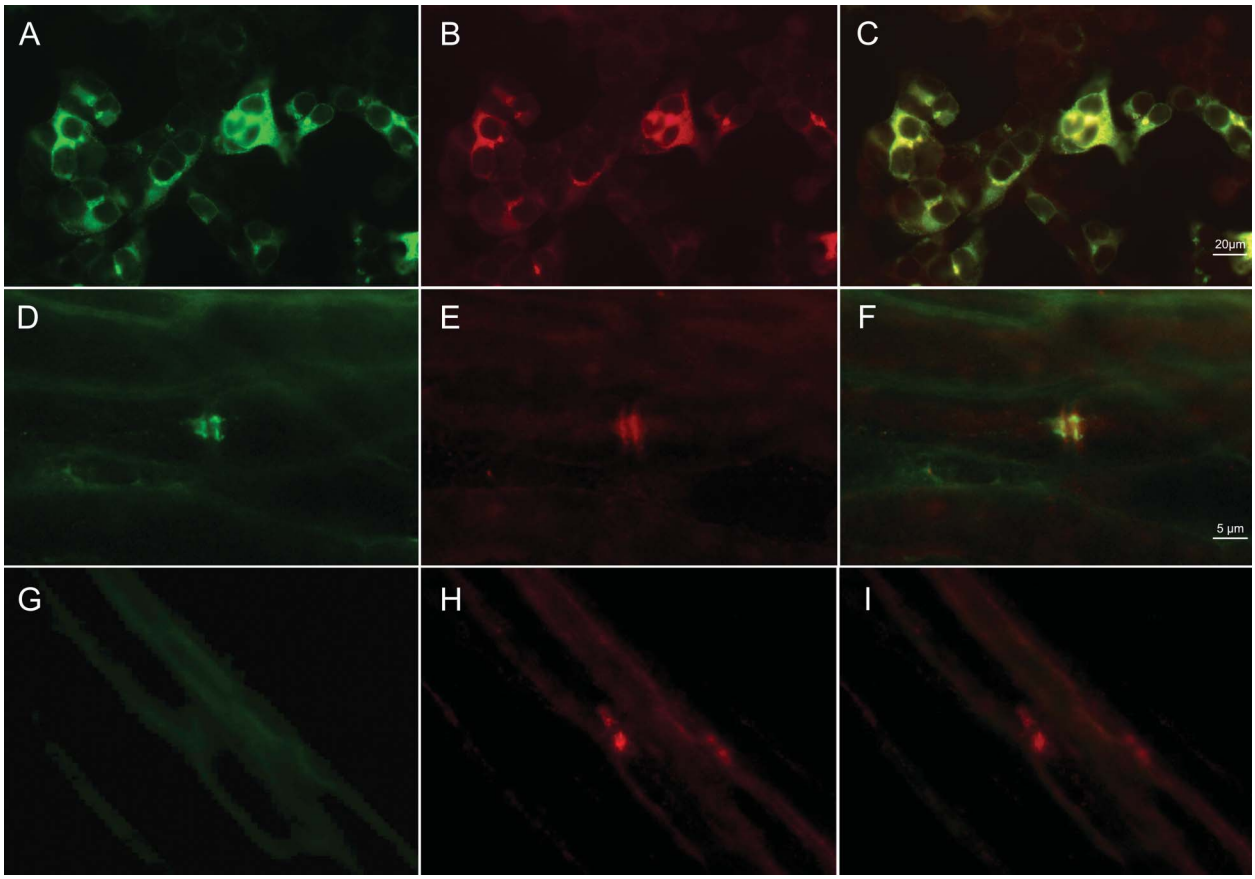
NFASC and the clinical features associated with these antibodies in patients with CIDP. The study provides Class IV evidence that antibodies against NF155 of the IgG4 isotype identify a specific subgroup of patients with CIDP who have severe polyradiculoneuropathy, poor response to IVIg, and disabling tremor.

RESULTS Using immunocytochemistry with NF155-transfected HEK cells, 2 of 53 patients followed in our unit (3.6%) had antibodies against NF155 (figure 1), whereas none of the 204 controls showed any reactivity ($p = 0.041$; Fisher exact test). Similar results were shown by ELISA ($p < 0.01$; figure 2A). None of the patients with CIDP or controls were positive for NF186 antibodies.

Patient 1 was a 46-year-old man who presented with distal weakness, paresthesias, and a low-frequency (3 Hz), high-amplitude postural and intention tremor (video 1). His symptoms progressed over 1 month and he was admitted to our hospital for further evaluation and treatment. Upper limb motor nerve conductions showed mildly decreased compound motor action potential (CMAP) amplitudes, very slow conduction velocities, prolonged distal motor latencies, temporal dispersion, and increased latency of F waves, without overt denervation in the needle EMG at onset. The patient was initially diagnosed with GBS and treated with IVIg without improvement. Corticosteroids were then started and, because of the lack of response, 6 PEx cycles were given with significant improvement of all symptoms, including the tremor. Corticosteroids and mycophenolate mofetil were used to prevent relapses. However, he developed 2 severe relapses with prominent distal weakness, hypoesthesia, ataxia, and postural and intention tremor. There was no response to additional treatment with IVIg, and PEx was started with significant improvement of all symptoms in both relapses. Persistent severe distal weakness, ataxia, and tremor that did not respond to immune therapies subsequently developed.

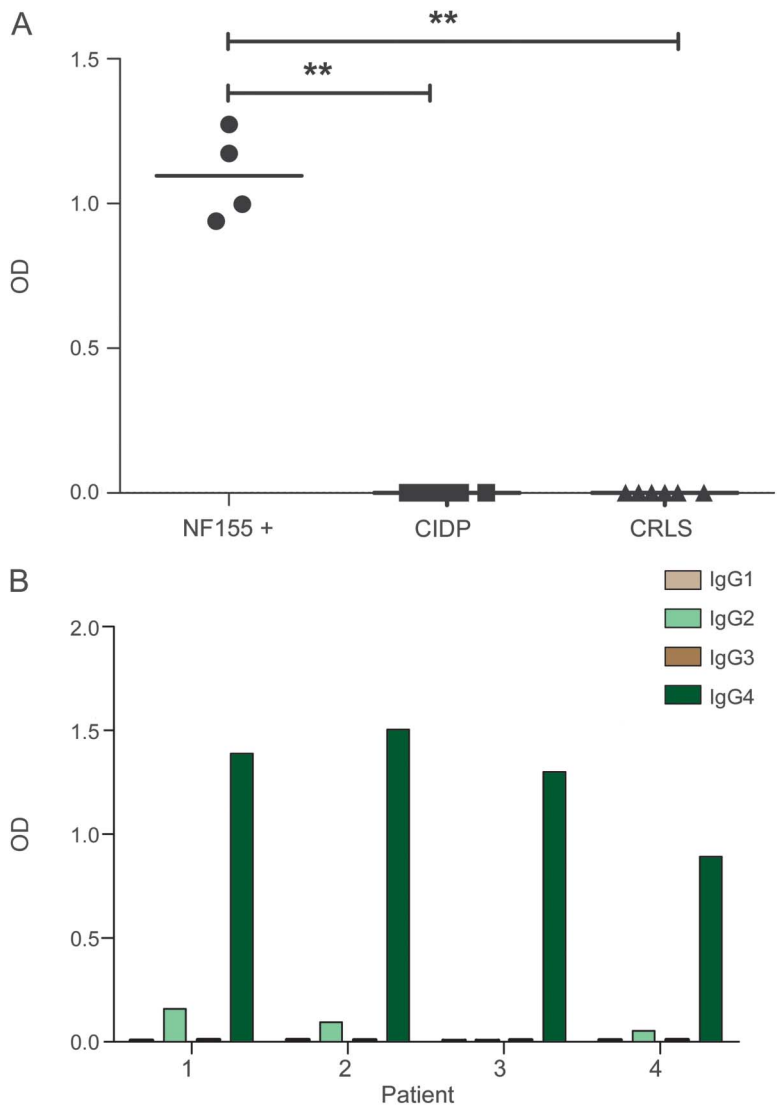
Patient 2 was a 25-year-old man who presented with a 1-month history of proximal and distal limb weakness that progressed to complete distal paralysis of the lower limbs in a few weeks. Distal dysesthesia, numbness, and ataxia appeared sequentially later on. A moderate low-frequency (6.6 Hz), low-amplitude postural and intention tremor, predominantly in the upper limbs, was detected. Motor neurography showed

Figure 1 **Immunocytochemistry and immunohistochemistry of anti-NF155+ patients**



(A–C) Commercial anti-neurofascin 155 (NF155) antibody (A) and patient 3 serum (B) signals colocalize (C) in immunocytochemistry using NF155-transfected HEK293 cells. (D–F) Commercial anti-NF155 antibody (D) and patient 1 serum (E) signals colocalize (F) at the paranode in immunohistochemistry using teased-nerve fibers. (G–I) Patient 2 serum reactivity against the paranode (G) is abrogated when it is preincubated with NF155-transfected HEK cells.

Figure 2 Comparison of reactivity against NF155 and anti-NF155 IgG isotypes measured using ELISA



Reactivity against NF155 measured using ELISA (A) was significantly different in patients with anti-NF155 antibodies (tested by immunocytochemistry) than in patients with CIDP without anti-NF155 antibodies ($p < 0.01$) or in normal controls ($p < 0.01$) at a serum dilution of 1:100. The predominant anti-NF155 IgG isotype in anti-NF155+ patients was IgG4 (B). CIDP = chronic inflammatory demyelinating polyradiculoneuropathy; CRLS = normal controls; IgG = immunoglobulin G; NF155 = neurofascin 155; NF155+ = CIDP patients with anti-NF155 antibodies detected by immunocytochemistry; OD = optical density.

decreased CMAP amplitudes, very slow conduction velocities, and prolonged distal and F-wave latencies without signs of denervation. A course of IVIg was tried with no response and oral corticosteroids were started to which he showed mild improvement. The patient was recently admitted and treated with 7 cycles of PEx that had so far resulted in moderate improvement.

Considering the unusual fact that both patients with NF155 antibodies were IVIg-resistant, we searched the Spanish CIBERNED-CIDP registry for other patients with CIDP who had no response to IVIg and identified 8 of 86 patients (8.3%). We evaluated all 8 for NFASC antibodies and found that 2 had anti-NF155 antibodies. Both of these patients presented with an aggressive, predominantly distal, motor and ataxic neuropathy similar to that of the 2 anti-NF155+ patients from our own cohort. One of these patients (patient 3) also had a very disabling, low-frequency (4 Hz), high-amplitude postural and intention tremor (video 2) that interfered with his daily activities. Initial motor nerve conduction studies showed a widespread decrease in CMAP amplitudes, prolonged distal latencies, and slowed conduction velocities, with no overt signs of denervation. Several IVIg courses were tried in patient 3 with no response. Other treatments such as prednisone and methotrexate were also tried with poor response. The CIDP features of patient 4 resembled those of the other 3, but no tremor was present. Five years after CIDP diagnosis, this patient presented with an IgM monoclonal gammopathy and a marginal zone B-cell lymphoma that responded to cyclophosphamide, rituximab, and prednisone. However, the CIDP did not improve despite aggressive immunotherapy for the marginal zone lymphoma and the patient became wheelchair-bound.

Brain MRI was performed in the 3 patients with tremor, showing no abnormalities. Brain dopamine-transporter scan was normal in patient 1 and was not performed in the other 3 patients. Clinical information on the 4 anti-NF155+ patients is summarized in table 1 and electrophysiologic data are shown in table e-2.

Anti-NF155 titers ranged from 1:8,000 in patient 4 to 1:70,000 in patients 1 and 2. In all 4 anti-NF155+ patients, IgG4 was the predominant IgG isotype, although anti-NF155 IgG2 was found at low concentrations in patients 1, 2, and 4 (figure 2B). Interestingly, patient 4 did not have IgM reactivity against NF155 despite the presence of the IgM monoclonal band. All 4 patients showed paranodal staining in teased-nerve immunohistochemistry (figure 1D) that colocalized with a commercially available NF155 polyclonal antibody (figure 1E).

The serum of all 4 patients showed intense reactivity against the neuropil of rat brain (including cortex, hippocampus, basal ganglia, cerebellum, and brainstem) that was not present in control sera. Despite this widespread reactivity, the pattern of staining of the hippocampus and cerebellum was identical for all 4 patients (figure 3). In the cerebellum, the reactivity predominated in the molecular and granular layers (figure 3, B and C).

Reactivity against the paranode was abrogated in all 4 patients when sera were preincubated with NF155-transfected HEK cells (figure 1, G–I) but not when absorption was performed with nontransfected HEK cells (data not shown).

DISCUSSION In our study, we examined the clinical features of CIDP associated with anti-NF155

Table 1 Epidemiologic, clinical, and electrophysiologic features of patients with CIDP positive for anti-NF155 antibodies

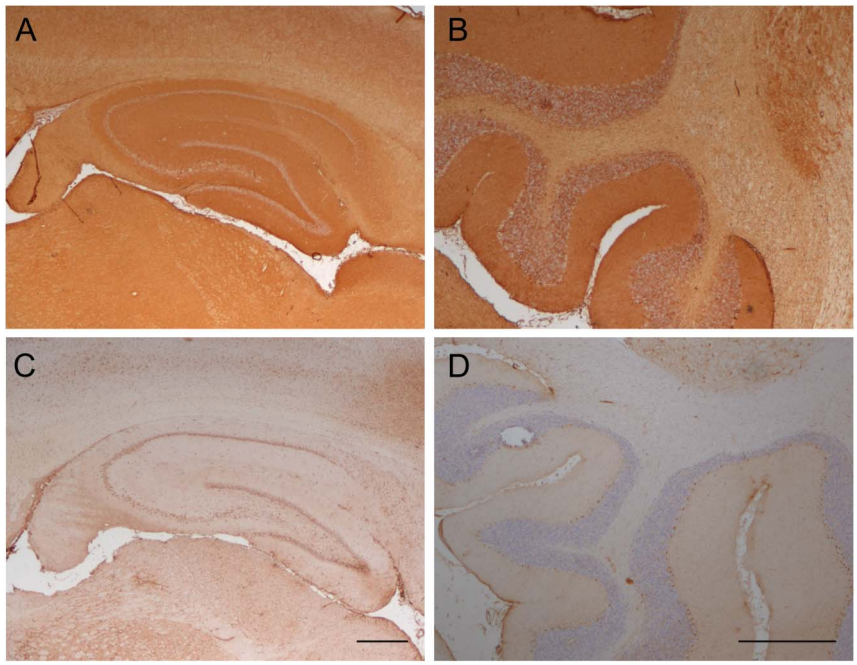
Patient	Age at onset (sex)	Symptoms	mRS score	CSF	Brain MRI	Treatments (responses)	Tremor frequency (amplitude)	NF155 titers
1	46 (M)	Rapidly progressive onset; severe weakness, predominantly distal; sensory disturbances; ataxia; severe intention tremor	4	1.5 g/L; 2 cells	Normal	IVIg (no); prednisone (no); PEx (yes)	3 Hz (9/10)	1:70,000
2	22 (M)	Chronic progressive; severe weakness, predominantly distal; sensory disturbances; ataxia; moderate intention tremor	4	4.6 g/L; 6 cells	Normal	IVIg (no); prednisone (partial); PEx (yes, partial)	6.6 Hz (2/10)	1:70,000
3	29 (M)	Chronic progressive; severe weakness, proximal and distal; sensory disturbances; ataxia; severe intention tremor	4	1.4 g/L; 7 cells	Normal	IVIg (no); prednisone (no); methotrexate (no)	4 Hz (8/10)	1:25,000
4	67 (F)	Chronic progressive; severe weakness, predominantly distal; sensory disturbances; no tremor	4	0.41 g/L; 0 cells	ND	IVIg (no); prednisone (no); cyclophosphamide (no); rituximab (no)	Not present	1:8,000

Abbreviations: CIDP = chronic inflammatory demyelinating polyradiculoneuropathy; IVIg = IV immunoglobulin; mRS = modified Rankin Scale; ND = not determined; NF155 = neurofascin 155; PEx = plasma exchange.

antibodies of the IgG4 subclass. All patients had a severe, predominantly distal, motor neuropathy with ataxia. Interestingly, 3 of 4 patients showed a striking low-frequency postural and intention tremor that was not present in any other patient with CIDP of our series. Our 4 patients had a neuropathy with prominent demyelinating features and no axonal degeneration in the initial EMG. None of the 4 patients responded to IVIg.

NF155 is the glial ligand of the CNTN1/CASPR1 complex at the paranode and thus has a key role in myelination and node of Ranvier functional organization. Additionally, selective knockout of the 155 isoform of neurofascin in mice causes a severe demyelinating neuropathy with a dramatic reduction in conduction velocities and degenerative changes in Purkinje neurons, leading to prominent tremor and ataxia.[25] These data

Figure 3 Demonstration of anti-neurofascin 155 antibodies in the serum of a patient with chronic inflammatory demyelinating polyradiculoneuropathy, disabling tremor, and poor response to IV immunoglobulin



The reactivity of the patient's serum antibodies using immunohistochemistry of rat brain is shown by brown staining in hippocampus (A) and in cerebellum (B). (C, D) Lack of reactivity of serum from an individual without neurofascin antibodies, who served as a control. Original magnification ×2 (A, C) and ×4 (B, D). Counterstained with hematoxylin. Bar = 500 μm.

suggest that anti-NF155 antibodies are likely to be pathogenic.

Of interest, our patients did not have typical proximal weakness. All 4 patients had predominant distal involvement of the upper and lower limbs and ataxia. The clinical course was slowly progressive in 2 patients (patients 3 and 4) and rapidly progressive in the other 2 (patients 1 and 2). In fact, patient 1 was initially classified as GBS because of the acute and aggressive onset. A similar acute onset was characteristically present in our patients with antibodies to CNTN1.[16]

A striking finding in the neurologic examination in 3 of our patients with NF155+ CIDP was the presence of a severe and disabling postural and intention tremor. No other patient from our cohort showed such a disabling tremor. In general, intention tremor is associated with lesions or diseases that involve the cerebellum or its outflow through the dentatorubrothalamic pathways.[26] The features of the tremor in our patients suggested a cerebellar origin although other signs of cerebellar impairment (nystagmus, oculomotor disturbances) were not present. Tremor is a frequent finding in patients with CIDP and some authors propose a cerebellar origin of the tremor in a significant proportion of patients with CIDP .[27,28] A recent report describes abnormal cerebellar function tests in the subset of patients with CIDP presenting with tremor that are not present in those without tremor, suggesting that the impairment of cerebellar function is related to the development of tremor in patients with CIDP.[28] The authors speculate that differences in the antigenic target of both subtypes could account for these differences. We demonstrate that the serum of anti-NF155+ patients react intensely with the neuropil of rat brain, with a pattern of immunostaining of hippocampus and cerebellum that was identical in all cases, suggesting that NF155 is one of the target antigens in patients with CIDP who have low-frequency, high amplitude tremor. One of our patients did not show a prominent tremor but showed a rat brain staining pattern similar to the other 3. This patient had lower anti-NF155 titers than the other 3 and this could influence the intrathecal levels of anti-NF155 antibodies and, thus, the presence of tremor. Nevertheless, further studies are needed to clarify whether anti-NF155 antibodies cause cerebellar pathology and whether these antibodies distinguish between CIDP patients with and without overt tremor of cerebellar origin.

Antibodies against NF155 have recently been described in CIDP, GBS, and MS, although the clinical phenotypes associated with these antibodies have not been described in detail.[13–15,20] In our series, anti-NF155 antibodies were found only in a subgroup of patients with CIDP and were not present in 204 controls, including 51 patients with GBS. Our control series, however, did not include patients with MS. Also, a high frequency of anti-NF155 antibodies has recently been reported in Asiatic patients with CCPD.[21] Despite the presence of a prominent tremor in 3 of our patients with some features suggesting a cerebellar origin, central demyelination was not found in any by MRI. Whether this phenotypic diversity of anti-NF155+ patients (GBS, MS, CIDP) is attributable to a difference in the antibodies (e.g., different IgG isotype), ethnic differences, or to selection biases will need to be clarified. None of our 4 anti-NF155 patients responded to IVIg, whereas patients with CCPD and anti-NF155 responded well.[21]

Anti-NF155 antibodies in our patients were predominantly of the IgG4 isotype. Antibodies of the IgG4 isotype do not activate complement or bind to Ig Fc domain receptors,[29] elements that are involved in IVIg action mechanisms.[30–32] This fact could account for the lack of response to IVIg. Although not all IVIg-resistant patients have anti-NF155 antibodies, it is interesting to note that the frequency of these antibodies increased from 3.6% in a conventional CIDP series (2/53 patients) to a much higher rate of 25% (2/8) when we selected for IVIg-resistant patients. In our CIDP series, that included patients referred from other centers; 11 of 42 IVIg-treated patients (26.1%) were classified as IVIg-resistant. Two of those eleven (18.18%) were anti-NF155+, results similar to those in the CIBERNED-CIDP cohort (25%).[22] These data suggest that anti-NF155 antibodies identify a substantial proportion of patients with IVIg-resistant CIDP and, thus, their identification has therapeutic and prognostic implications.

The search for autoantibodies in CIDP has been a topic of research for years. Recently, our group and others have shown that the node of Ranvier and related structures are the target of the immune response in a subgroup of patients with CIDP; antibodies against nodal and paranodal proteins, such as CNTN1, NF155, NF186, gliomedin, and NrCAM are present in small subgroups of patients with CIDP.[12,13,15,16] CIDP heterogeneity is currently determined by different electrophysiologic degrees of CIDP certainty and the inclusion of atypical variants in the diagnostic criteria.[2] The clinical features associated with anti-CNTN1 that we previously reported[16] and those associated with anti-NF155 reported here suggest that CIDP subtypes will become more homogeneous when they are defined by autoantibody status. The main practical implication of the current study is that NF155 antibodies identify a CIDP phenotype characterized by severe polyradiculoneuropathy, poor response to IVIg, and disabling tremor. This association has been established based on a limited number of patients and should be confirmed in larger cohorts.

AUTHOR CONTRIBUTIONS

Conceived, designed, and performed the experiments: L.Q., G.N.-G., E.G., J.D., I.I. Analyzed the data: L.Q., G.N.-G., E.G., J.D., R.B., I.I. Wrote and edited the manuscript: L.Q., G.N.-G., J.D., R.B., I.I. Provided characterized specimens: J.D.-M., R.R.-G., J.P., J.B., M.J.S., A.O.-M.

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DISCLOSURE

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