Lack of KIR4.1 autoantibodies in Japanese patients with MS and NMO

ABSTRACT

Objectives: To examine anti-KIR4.1 antibodies by 2 different assays in Japanese patients with multiple sclerosis (MS) or neuromyelitis optica (NMO).

Methods: One hundred sixty serum samples from 57 patients with MS, 40 patients with NMO/NMOSD, and 50 healthy controls (all were Japanese) were tested with ELISA using a synthetic peptide of the first extracellular portion of human KIR4.1. In addition, we attempted to detect anti-KIR4.1 immunoglobulin G in the serum by the luciferase immunoprecipitation systems (LIPS) with the full length of human KIR4.1 produced in a human cell line, which is highly sensitive to single or multiple epitopes.

Results: We failed to detect antibodies to the peptide fragment KIR4.183–120 in any case of MS and NMO/NMOSD using ELISA. Antibodies to the recombinant full length of KIR4.1 protein were detected in only 2 patients with MS and none in the patients with NMO/NMOSD by the LIPS assay.

Conclusions: We developed 2 different methods (ELISA and LIPS) to measure autoantibodies to KIR4.1 in serum. We detected anti-KIR4.1 immunoglobulin G at a very low frequency in Japanese patients with MS or NMO/NMOSD. Serologic testing for human KIR4.1-specific antibodies is unlikely to improve the diagnosis of MS or NMO/NMOSD in Japanese patients.

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GLOSSARY

AQP4 = aquaporin-4; GL = Gaussia luciferase; IgG = immunoglobulin G; KIR4.1 = inward rectifying potassium channel 4.1; LIPS = luciferase immunoprecipitation systems; MS = multiple sclerosis; NMO = neuromyelitis optica; NMOSD = neuromyelitis optica spectrum disorder; PBS = phosphate-buffered saline; PBST = phosphate-buffered saline with Tween 20; RLU = relative luminescence units.

The causes and disease pathways of multiple sclerosis (MS) remain poorly understood; especially unclear is the role of B cells in the pathogenesis of MS. Oligoclonal bands are recognized as a key immunopathologic feature of MS and other neuroinflammatory diseases.

Neuromyelitis optica (NMO) has been regarded as a variant of MS, but with demonstrated distinctive pathologic features. It is another devastating CNS demyelinating disease characterized by severe optic neuritis and transverse, longitudinally extensive myelitis. The discovery of a pathogenic antibody against the astrocyte water channel protein aquaporin-4 (AQP4), with a high diagnostic sensitivity and specificity for NMO, indicates that this condition is distinct from MS.

Srivastava et al.1 reported that antibodies against the inward rectifying potassium channel 4.1 (KIR4.1) were detected in the serum of patients with MS. The authors found anti-KIR4.1 antibodies in serum samples from 47% of patients with MS, 1% with other neurologic diseases,
and in none of the healthy controls. Accordingly, KIR4.1 is a candidate pathogenic autoantigen in MS, but subsequent studies have not confirmed the association.2,3 In addition, the coexpression of KIR4.1 and AQP4 channels on astrocyte endfeet may suggest a relationship between anti-KIR4.1 antibodies and NMO/NMOSD.

In this study, we used 2 different assays to measure autoantibodies to KIR4.1 in serum from Japanese patients with MS and NMO, and investigated the clinical features of seropositive patients in Japan. In addition, we have recently developed the luciferase immunoprecipitation systems (LIPS) assay to measure antibodies to KIR4.1, which can detect protein–protein interactions with high sensitivity.4–6

METHODS Standard protocol approvals, registrations, and patient consents. The ethics committee of Nagasaki Kawatana Medical Center (Nagasaki, Japan) approved this study. Informed consent was obtained from each participant, in person, before participation in the study. Participants provided written informed consent, and this process was documented on an approved consent form.

Patients and controls. Fifty-seven patients with MS were recruited from the Hokkaido Medical Center and Sapporo Neurology Clinic. Recruitment was limited to patients who were not receiving disease-modifying therapy. The diagnosis of MS was confirmed using 2005 and 2010 revisions to the McDonald criteria.2,3 Patient demographic data were as follows: median age, 40.6 ± 11.5 years; male/female ratio, 11/46; onset age, 27.8 ± 8.4 years; and Expanded Disability Status Scale score, 2.7 ± 2.3. Patients were classified as having relapsing-remitting MS (n = 11), secondary progressive MS (n = 11), or an unclassified (n = 1) disease course by 2 trained neurologists. Clinical sampling phases were as follows: onset = 2; relapse = 16; remission = 30; clinically isolated syndrome = 1; unclassified = 11.

Forty patients with NMO/NMOSD were recruited from Tohoku University Graduate School of Medicine. Recruitment was limited to patients who were seropositive for anti-AQP4 antibodies.9 The diagnosis of NMO/NMOSD was confirmed using the revised NMO criteria.10 Patient demographic data were as follows: median age, 40.6 ± 14.3 years; male/female ratio, 1/39; onset age, 43.0 ± 13.7 years; and NMO/NMOSD, 21/19. Clinical sampling phases were as follows: acute phase = 1; relapse = 12; chronic phase = 27. Thirty-six of 40 patients with NMO/NMOSD were administered oral prednisolone.

An additional 50 serum samples from healthy controls were tested (mean age, 35.5 ± 9.2 years, 11 men and 39 women). They were recruited from the Nagasaki Kawatana Medical Center. All 160 serum samples were obtained separately from 2002 to 2013 at 3 locations and cryopreserved at −80°C until use in this study.

ELISA system using the 38-mer synthetic peptide of human KIR4.1. According to the method described,1 a 38-mer peptide (NH₂-G-Y-V-W-Y-L-V-A-V-A-H-G-D-L-L-E-L-D-P-P-A-N-H-T-P-C-V-Q-V-H-T-L-T-G-A-F-L-COOH) representing the extracellular loop and adjacent intramembrane regions of human KIR4.1 was synthesized and its amino terminus was tagged with biotin (Peptide Institute, Inc., Osaka, Japan). The peptide (10 μg/mL) was dissolved in phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20 (PBST), and 100 μL of the peptide solution was layered onto streptavidin-coated microtiter plates (Nunc, Roskilde, Denmark) at room temperature for 3 hours. After blocking the wells with PBST supplemented with 3% bovine serum albumin, 100 μL of diluted human sera at 100-fold with PBST was added to each well and allowed to react with the peptide at 4°C overnight. After thoroughly washing the wells with PBST, 100 μL of 1:2,500 goat anti-human immunoglobulin G (IgG) (H+L) antibody horseradish peroxidase conjugate (Promega, Madison, WI) was added and incubated for 1 hour at room temperature. After extensive washing, 100 μL of the substrate solution in the TMB Peroxidase ELISA Substrate Kit (Bio-Rad Laboratories, Hercules, CA) was added, and the reaction was stopped by adding 100 μL of 1 N H₂SO₄. A microplate reader was used to measure the optical density at 450 nm. Empty wells were used as a negative control in this assay. The control values were subtracted from the KIR4.1-derived peptide-speciﬁc values. ELISA assays were independently performed 3 times. As a positive control for the system, antiserum was prepared from a rabbit immunized with a synthetic peptide of human KIR4.1 (amino acids [aa] 92–105) (Peptide Institute, Inc., Osaka, Japan). Preimmune serum was also collected from the same animal before immunization with the peptide (Peptide Institute, Inc.).

LIPS assay for full-length human KIR4.1. For LIPS, Gaussia luciferase (GL) was used as a luciferase reporter. Briefly, the LIPS procedure for human KIR4.1 was as follows. A GL reporter for a human KIR4.1 (KIR4.1-GL) expression plasmid was constructed with both human cDNAs encoding the full-length KIR4.1 (GenBank accession no. AB384828) and GL (from pGLuc-Basic; New England Biolabs, Ipswich, MA) in pcDNA3.1-Myc/His (A) (Invitrogen, Carlsbad, CA). The KIR4.1-GL expression plasmid was transfected to human 293F cells (Invitrogen) with FuGENE6 (Promega). Two days later, transfected cells cultured on a 100-mm culture dish were solubilized with 1 mL of lysis buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl; and 1% Triton X-100), and a soluble fraction was prepared as a GL reporter sample. Next, 100 μL of the reporter fraction and 15 μL of human serum were mixed for 1 hour at 4°C. To the mixture, 600 μL of PBS supplemented with 0.05% Tween 20, 3% bovine serum albumin, and 15 μL (volume of resin) of Protein G Sepharose (GE Healthcare, Little Chalfont, UK) was added; the mixture was then incubated for 1 hour at 4°C with rotation. The Protein G Sepharose was precipitated at a relative centrifugal force of 9,391 for a few seconds and extensively washed with PBST up to 2 times. A 50-μL substrate solution was added to the precipitate, and bioluminescence was measured by a Lumat LB 9507 Luminometer (Berthold Technologies, Zug, Switzerland). The luminoimeter output was expressed in relative luminescence units (RLU). Rabbit antiserum against a synthetic peptide of human KIR4.1 (residues 92–105) was used as a positive control to confirm the results of the LIPS assay. A cutoff value was calculated as the mean + 4 SDs based on the measurement values of healthy controls (n = 50). LIPS assays were independently performed 3 times.

Statistical analysis. Commercially available statistics software was used for data analysis (SigmaPlot; HULINKS Inc., Tokyo, Japan). One-way analysis of variance was used for comparisons of antibody titers in the LIPS test and optical density values in the ELISA between the healthy controls and patient groups.
RESULTS

Detection of autoantibodies against KIR4.1 in patients with MS and NMO/NMOSD using ELISA.

To verify the high clinical sensitivity for anti-KIR4.1 antibodies reported in MS,1 we used the ELISA system with a synthetic 38-mer peptide (aa 83–120 of human KIR4.1). This peptide is highly insoluble, perhaps because of its clusters of hydrophobic amino acids at both ends. Accordingly, one of our concerns was whether the wells in a microtiter plate were properly coated with the peptide. To confirm that 38-mer synthetic peptides bind in the wells of a microtiter plate, we examined the binding of a rabbit antiserum raised against an internal portion (aa 92–105) of human KIR4.1 to the 38-mer synthetic peptide-coated microtiter plate. As shown in figure 1A, anti-KIR4.1 92–105 rabbit antiserum, but not its preimmune serum, bound to the plate in a dose-dependent manner (figure 1A). Based on the cutoff value established from data for healthy controls, anti-KIR4.1 antibodies were not detected in any cases of MS or NMO/NMOSD (figure 1B).

Detection of autoantibodies to KIR4.1 using LIPS for patients with MS and NMO/NMOSD. To search for epitopes in the full-length human KIR4.1 polypeptide, we used the LIPS for the anti-KIR4.1 antibody. It has been demonstrated that LIPS is effective for detecting antibodies against several membrane proteins.2–4 To confirm the validity of the LIPS assay for anti-human KIR4.1 antibodies, anti-KIR4.1 92–105 rabbit antiserum for KIR4.1 was used as a positive control (figure 2A). The anti-KIR4.1 92–105 rabbit antiserum bound KIR4.1-GL in a dose-response manner, but not in preimmune serum, suggesting that anti-KIR4.1 antibodies in rabbit antiserum recognize the first extracellular portion in the full-length human KIR4.1 fused to GL. As shown in figure 2B, based on a cutoff of 0.708 × 10^5 RLU (mean + 4 SDs of 50 serum samples from healthy controls), only 2 patients with MS were positive for the anti-KIR4.1 antibody based on the LIPS assay (3.3%), while none of the patients with NMO/NMOSD or the healthy controls were autoantibody-positive. There were no statistically significant differences among the 3 groups (p = 0.278). To exclude the possibility that an antibody binding to GL is detected in this assay, we further performed LIPS using both KIR4.1-GL and GL. Two KIR4.1 antibody-positive sera of patients with MS bound to KIR4.1-GL in a dose-dependent manner. Conversely, the binding activities of these sera to GL were apparently lower than those to KIR4.1-GL, indicating that immunoglobulin G binding KIR4.1 was contained in the sera of 2 patients with MS (figure e-1 at Neurology.org/nn).

Clinical profile of anti-KIR4.1 antibody–positive patients with MS or NMO/NMOSD. The table summarizes the clinical profiles of 2 anti-KIR4.1 antibody–positive patients with MS or NMO/NMOSD. The table summarizes the clinical profiles of 2 anti-KIR4.1 antibody–positive patients with MS or NMO/NMOSD.

![Figure 1 Detection of KIR4.1 autoantibodies using ELISA with the synthetic peptide](Neurology.org/nn)
patients with MS. The patient characteristics were rather typical of MS (patients 1 and 2). We found no specific clinical features in these patients other than the basic disease.

**DISCUSSION** It had been reported that there was a strong correlation between the serum antibody reactivities measured by ELISA with the recombinant full length of KIR4.1 and measured by ELISA with the peptide fragment KIR4.1\textsuperscript{83-120}.\textsuperscript{1} In accordance with their findings, we also used the 2 types of antigens to measure serum antibody to KIR4.1. One was the peptide fragment KIR4.1\textsuperscript{83-120} designed by a previous study for ELISA.\textsuperscript{1} Another was the recombinant full length of KIR4.1 synthesized in a human cell line for LIPS. In the ELISA method, we failed to detect anti-KIR4.1 IgG in Japanese patients with MS and NMO/NMOSD. Meanwhile, anti-KIR4.1 antibody positivity was detected in 2 patients with MS using the LIPS method. In the LIPS, recombinant proteins are produced in human cells and are directly tagged with the highly sensitive GL reporter enzyme. One of the key advantages of our LIPS test is the large dynamic range of detection of antibodies because of the solution-phase assay format and the high signal-to-noise ratio of the GL reporter. These results differ markedly from those of the previous report\textsuperscript{1} in which KIR4.1 was identified as a candidate autoantigen in MS. However, the results of this study were consistent with the low prevalence of anti-KIR4.1 antibodies observed in patients with MS by several groups.\textsuperscript{2,3} By contrast, the other group recently detected anti-KIR4.1 antibodies in the serum of patients with MS and NMO, at a significantly higher frequency.

**Table** Clinical features of anti-KIR4.1 Ab-positive patients with MS

|                      | Patient 1 | Patient 2 |
|----------------------|-----------|-----------|
| Measuring Ab for KIR4.1 with ELISA | –         | –         |
| Measuring Ab for KIR4.1 with LIPS assay | +         | +         |
| Age, y               | 40        | 34        |
| Sex                  | Female    | Female    |
| Onset age, y         | 27        | 20        |
| Subtype              | RRMS      | RRMS      |
| Condition at sampling | Remission | Remission |
| Symptom at onset     | Sensory disturbance | Sensory disturbance |
| No. of relapses      | 9         | 4         |
| Latest EDSS score    | 3.0       | 1.0       |
| MS lesions           | Brain, bilateral optic nerves, spinal cord | Optic nerve (right), cervical cord |
| MRI findings         | Brain, corpus callosum, spinal cord | Brain, corpus callosum, spinal cord |
| Oligoclonal bands    | Positive  | Positive  |

Abbreviations: Ab = antibody; EDSS = Expanded Disability Status Scale; KIR4.1 = inward rectifying potassium channel 4.1; LIPS = luciferase immunoprecipitation systems; MS = multiple sclerosis; RRMS = relapsing-remitting MS.
and at higher levels compared to healthy controls. This discrepancy is difficult to explain since analogous ELISA techniques used in each study were not identical and the samples were ethnically different. To clarify the differences, it is necessary to perform various assays to detect KIR4.1 IgG using the same coded samples in an international multicenter study.

KIR4.1 is an inwardly rectifying potassium channel for the osmotic homeostasis of extracellular potassium, which is expressed on oligodendrocytes and the perivascular and perisynaptic endfeet of astrocytes. It is localized with AQP4 at the interface of astrocytes and blood vessels, where KIR4.1 cooperates with AQP4 for the balance of potassium and water transport. This suggests a functional link between the 2 channels. Several studies suggested NMO as an autoimmune astrocytopathy in which damage to astrocytes exceeds both myelin and neuronal damage. We also studied 50 samples from patients residing in other regions, including the northern part of Japan. Therefore, we collected sera from patients with MS residing at 2 hospitals located at high altitudes, where the incidence of MS is the highest in Japan. Taken together, serologic testing for anti-KIR4.1 antibodies is unlikely to aid in the diagnosis of MS or NMO/NMOSD in a Japanese population.

The other research group in Japan already screened for anti-KIR4.1 antibodies using ELISA in a cohort of Japanese patients with idiopathic CNS demyelinating disease. They also showed that anti-KIR4.1 antibodies are not specific to MS; the antibody positivity rate in MS was only 3.9% for 180 patients with MS, which is much lower than the 46.9% previously described. They mentioned a need for replication studies using Japanese patients residing in other regions, including the northern part of Japan. Therefore, we collected sera from patients with MS residing at 2 hospitals located at high altitudes, where the incidence of MS is the highest in Japan. Taken together, serologic testing for anti-KIR4.1 antibodies is unlikely to aid in the diagnosis of MS or NMO/NMOSD in a Japanese population.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: O.H., S.N., M.N., K.F., H.M. Performed the experiments: O.H., W.S., Y.M. Analyzed the data: O.H., S.N., M.N., T.T., T.F., S.K., K.F., H.M. Wrote the manuscript: O.H., S.N., M.N., K.F., H.M.

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