Rabphilin-3A as a Targeted Autoantigen in Lymphocytic Infundibulo-neurohypophysitis

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Context: Central diabetes insipidus (CDI) can be caused by several diseases, but in about half of the patients the etiological diagnosis remains unknown. Lymphocytic infundibulo-neurohypophysitis (LINH) is an increasingly recognized entity among cases of idiopathic CDI; however, the differential diagnosis from other pituitary diseases including tumors can be difficult because of similar clinical and radiological manifestations. The definite diagnosis of LINH requires invasive pituitary biopsy.

Objective: The study was designed to identify the autoantigen(s) in LINH and thus develop a diagnostic test based on serum autoantibodies.

Design: Rat posterior pituitary lysate was immunoprecipitated with IgGs purified from the sera of patients with LINH or control subjects. The immunoprecipitates were subjected to liquid chromatography-tandem mass spectrometry to screen for pituitary autoantigens of LINH. Subsequently, we made recombinant proteins of candidate autoantigens and analyzed autoantibodies in serum by Western blotting.

Results: Rabphilin-3A proved to be the most diagnostically useful autoantigen. Anti-rabphilin-3A antibodies were detected in 22 of the 29 (76%) patients (including 4 of the 4 biopsy-proven samples) with LINH and 2 of 18 (11.1%) patients with biopsy-proven lymphocytic adeno-hypophysitis. In contrast, these antibodies were absent in patients with biopsy-proven sellar/suprasellar masses without lymphocytic hypophysitis (n = 34), including 18 patients with CDI. Rabphilin-3A was expressed in posterior pituitary and hypothalamic vasopressin neurons but not anterior pituitary.

Conclusions: These results suggest that rabphilin-3A is a major autoantigen in LINH. Autoantibodies to rabphilin-3A may serve as a biomarker for the diagnosis of LINH and be useful for the differential diagnosis in patients with CDI. (***J Clin Endocrinol Metab*** **100: E946–E954, 2015)**

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Abbreviations: AH, autoimmune hypophysitis; AVP, arginine vasopressin; AVPc-Ab, vasopressin-cell autoantibody; BSA, bovine serum albumin; CDI, central diabetes insipidus; DI, diabetes insipidus; LAH, lymphocytic adeno-hypophysitis; LINH, lymphocytic infundibulo-neurohypophysitis; LPH, lymphocytic pan-hypophysitis; LC, liquid chromatography; MRI, magnetic resonance imaging; MS/MS, tandem mass spectrometry; NLGN1, neuroligin-1; OTOF, otoferlin; rh, recombinant human; RIMS1, regulating synaptic membrane exocytosis protein 1; RPH3A, rabphilin-3A; SCG2, secretogranin II; USO1 general vesicular transport factor p115.

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Central diabetes insipidus (CDI) representing polyuria due to insufficient arginine vasopressin (AVP) secretion can be caused by several diseases (eg, tumors, trauma, or inflammation), but in about half of the patients the etiological diagnosis remains unknown (1). An important cause among them is autoimmune hypophysitis (AH), which is a chronic form of inflammation of the pituitary gland with varying degrees of pituitary dysfunction (2), featuring an infiltration of hematopoietic cells (mainly lymphocytes) that causes initial expansion and ultimate atrophy of the pituitary (3). Depending on the infiltrated cell types, AH is histologically classified into lymphocytic, granulomatous, xanthomatous, IgG4-plasmacytic, or necrotizing forms, among which more than 70% are lymphocytic (4). In lymphocytic hypophysitis, there are 3 forms that are classified according to the location of the hematopoietic infiltrate: lymphocytic adeno-hypophysitis (LAH), lymphocytic infundibulo-neurohypophysitis (LINH), and lymphocytic pan-hypophysitis (LPH) (5). LINH, in which lymphocytes infiltrate the neurohypophysis, causes CDI and a swelling of posterior pituitary and/or stalk.

Clinically, the differential diagnosis of LINH and other pituitary diseases that cause CDI, such as sellar or suprasellar tumors (eg, Rathke cleft cyst, craniopharyngioma, or germinoma), can be difficult because of the similar clinical presentation and radiographic appearance. Headache and visual disturbances associated with mass effects are common manifestations of LINH as well as tumors. Although magnetic resonance imaging (MRI) is useful for the diagnosis of LINH, swelling of the pituitary and/or stalk and enhancement by contrast agents are also observed in tumors. Di Iorgi et al (1) reported that long-term follow-up unmasked a diagnostic etiology in some patients with childhood-onset CDI with stalk abnormalities, but it is difficult to clarify it at the beginning.

The pathogenesis of LINH and the autoantigen(s) involved remains poorly characterized, even though LINH was first reported approximately 40 years ago (6). LINH is thought to be a major cause of idiopathic CDI (7). Many candidate autoantigens in LAH, which was first reported (8) prior to LINH, have been proposed since 2001 (9–14), although none have entered the clinical arena. Autoantigen identification has clinical significance because such findings may lead to the development of diagnostic tests based on serum autoantibodies that could distinguish AH from other diseases without the need for invasive transsphenoidal pituitary biopsy. Autoantigen identification would also provide important insight into the pathology of AH.

Technological innovations in proteomics using high-throughput mass spectrometry have enabled the exhaustive analysis of candidate autoantigens in autoimmune diseases (15–17). To identify autoantigen(s) in LINH, we used shotgun liquid chromatography (LC)-tandem mass spectrometry (MS/MS) on immunoprecipitates obtained from patient sera incubated with posterior pituitary protein lysate, and the results revealed that rabphilin-3A is an autoantigen in LINH. We further validated this autoantibody as a novel diagnostic marker.

Subjects and Methods

Patient features

The study included 223 serum samples. The samples were obtained from Nagoya University, other facilities in Japan, and Johns Hopkins University, following approval of an institutional review board protocol in each facility. All patients signed a written informed consent form. The biopsy-proven LINH (n = 4) or LAH (n = 18) samples were diagnosed based on the pathological findings, including extensive lymphocytic infiltration with predominance of T cells in the infundibulum, the stalk, and the neurohypophysis or the anterior pituitary gland, respectively. To determine the clinical diagnosis of LINH (n = 25), we used the following criteria: criterion 1: clinical symptoms and laboratory data indicating the presence of CDI; and criterion 2: MRI findings showing swelling of the pituitary gland and/or stalk with high enhancement of the pituitary lesion in contrast-enhanced MRI with gadolinium. Criteria 1 and 2 were fulfilled for the clinical diagnosis. Reference findings were as follows: (1) spontaneous improvement or disappearance of swelling of the pituitary gland and/or stalk on radiologic examination; and (2) normal levels of α-fetoprotein and β-human chorionic gonadotropin (tested if cerebrospinal fluid was available). A total of 34 biopsy-proven samples from sellar/suprasellar masses, including Rathke cleft cyst (n = 7), craniopharyngioma (n = 11), germinoma (n = 5), nonfunctioning pituitary adenoma (n = 5), Langerhans cell histiocytosis (n = 1), sarcoidosis (n = 1), glioma (n = 1), pituitary teratoma (n = 1), hypertrophic pachymeningitis (n = 1), and hypothalamic tumor (n = 1), were diagnosed based on the pathological findings for the surgical specimens. In addition, 31 patients with idiopathic CDI who showed no abnormal findings on MRI scans were included in this study. As controls, we used 70 patients with other autoimmune diseases without pituitary diseases (rheumatoid arthritis [n = 20], systemic lupus erythematosus [n = 15], progressive systemic sclerosis [n = 9], mixed connective tissue disease [n = 2], Sjögren's syndrome [n = 10], autoimmune thyroid diseases [n = 6], and polymyositis/dermatomyositis [n = 8]) as well as 41 healthy blood donors.

Immunoprecipitation shotgun LC-MS/MS

To purify IgGs, 200 μL of serum from the 3 patients with LINH (1 with biopsy-proven LINH and the other 2 with convincing LINH as diagnosed clinically) or healthy control subjects was incubated on a shaker with protein A beads (Byzen A; Nomadic Bioscience) after dilution with 800 μL of lysis buffer containing 20 mM Tris/HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, Complete protease inhibitor cocktail (Roche), and PhosSTOP phosphatase inhibitor cocktail (Roche) for 1.5 hours at 4°C. After the beads were washed 3 times with lysis buffer, the IgGs were eluted from the beads into 200 μL of lysis buffer by incu-

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bation at 40°C for 5 minutes. To prepare rat posterior pituitary lysate, 15 posterior pituitaries were collected from male Sprague-Dawley rats for each sample and homogenized with 150 μL of lysis buffer. The lysate was mildly reduced by incubation with 1 mM dithiothreitol at 50°C for 30 minutes, diluted 10 times, and centrifuged at 43 000 rpm for 1 hour. Then, 1.2 mL of supernatant was incubated on a shaker overnight at 4°C with the IgGs prepared as above and protein G magnetic beads (Dynabeads Protein G; Invitrogen). After the magnetic beads were washed 3 times with lysis buffer, proteins bound to the IgG magnetic beads were eluted by adding 90 μL of 500 mM NaCl. For protein reduction and alkylation, the samples were mixed with 133 mg of guanidine hydrochloride dissolved with 100 μL of water and then with 20 μL of 3 M Tris/HCl (pH 8.5), followed by 10 μL of 100 mM dithiothreitol. After a 30-minute incubation at room temperature, 10 μL of freshly prepared 200 mM iodoacetamide solution was added for 1 hour in the dark. For desalting and concentration, samples were mixed with 600 μL of methanol, 150 μL of chloroform, and 450 μL of water; the samples were then centrifuged, and the supernatant was removed. After mixing again with 450 μL of methanol, the supernatant was collected from the samples and dried under vacuum. Then, 0.5 μL of 1.0 μg/μL trypsin solution (Promega V5280) was added to the dried samples for protein in-solution digestion at 37°C overnight after mixing with 6 M urea and 40 μL of 0.1 M Tris/HCl (pH 8.5). The digested samples (50 μL) were directly analyzed on an OrbitrapXL mass spectrometer (Thermo Fisher Scientific).

Mass spectrometry analysis and data interpretation

For each sample, mass spectrometry analysis was performed as described previously (18). An LTQ Orbitrap XL mass spectrometry system (Thermo Fisher Scientific Inc.) combined with a Paradigm MS4 HPLC system (Michrom BioResources Inc.) was used for nanoelectrospray tandem mass analysis. Samples were injected onto the Paradigm MS4 HPLC system equipped with a Magic C18 AQ column 0.1 mm in diameter and 50 mm in length (Michrom BioResources Inc.). Reverse-phase chromatography was performed with a linear gradient (0 minutes, 5% B; 100 minutes, 50% B) of solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (90% acetonitrile with 0.1% formic acid) at an estimated flow rate of 1 μL/min. Ionization was performed by an ADVANCE Spray Source (Michrom BioResources Inc.) with a capillary voltage at 1.7 kV and temperature of 150°C. A precursor ion scan was performed using a 400 to 2000 mass to charge ratio (m/z) before MS/MS analysis. Multiple MS/MS spectra were submitted to the Mascot program (Matrix Science Inc.) for the MS/MS ion search. We considered proteins as candidates for autoantigens if they were detected in the LINH patient samples but not in those from healthy control subjects. We repeated the experiment at least 2 times.

Recombinant proteins

Vectors containing the full-length cDNA of human rabphilin-3A (hRPH3A) (MHS1011-76735), mouse otoferlin (mOTOF) (EMM1002-99866630), human neurolignin-1 (hNLGN1) (MHS1010-7508490), human general vesicular transport factor p115 (hUSO1) (pCMV-SPORT), mouse regulating synaptic membrane exocytosis protein 1 (mRIMS1) (OMM4760-99863456), and human secretogranin II (hSCG2) (IHS1380-97652155) were purchased from Open Biosystems, Inc. Each open reading frame was amplified by PCR, inserted into the expression vector pcDNA3.1D/V5-His-TOPO (Invitrogen), and completely sequenced using the sequencer (3130 Genetic Analyzer; Applied Biosystems). HEK293FT cells cultured in 10-cm dishes were transfected with each vector or an empty vector control using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 48 hours, transfected HEK293FT cells were collected with 1 mL of 1× sample buffer as a total cell lysate. Expression of each recombinant protein was confirmed by Western blotting with an anti-V5 antibody (Invitrogen).

Immunocytochemistry

Twenty-four hours after transfection with a vector containing RPH3A cDNA or empty vector, transfected HEK293FT cells were mixed with untransfected HEK293FT cells and then passed into a 3.5-cm glass-bottom dish at 20% to 25% confluence. After a 24-hour incubation, the cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. After being washed with PBS, the cells were incubated with 0.1% Triton X-100 for 10 minutes followed by an incubation with both patient serum (1:50) and anti-V5 antibodies (1:200). To examine the recognition of rabphilin-3A by patient serum, cells were stained with Alexa Fluor 488–conjugated anti-human IgG and Alexa Fluor 594–conjugated anti-mouse IgG. Colocalization was observed with a fluorescence microscope (BZ-8000; Keyence).

Western blotting

Anterior or posterior pituitaries from Sprague-Dawley rats and human autopsy samples were homogenized on ice in radioimmunoprecipitation assay buffer (R0278; Sigma-Aldrich) supplemented with a cocktail of protease inhibitors (P8340; Sigma-Aldrich). Homogenates were cleared by centrifugation at 600 × g to pellet insoluble debris. Supernatants were then aliquoted and stored at −80°C. Ten micrograms of the protein lysates were separated on 7.5% SDS-PAGE gels under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were then blocked in 4% skim milk and incubated with anti-rabphilin-3A antibodies overnight at 4°C. After 3 washes in PBS with Tween 20, membranes were incubated for 1 hour at room temperature with a peroxidase-conjugated secondary antibody (DAKO). Subsequently, blots were developed with the ECL Plus Western blotting detection reagent (GE Healthcare) and exposed to X-ray films.

Evaluation for the presence of anti-rabphilin-3A antibody in patient sera

To measure the presence of anti-rabphilin-3A antibodies in patient serum, 20 μL of recombinant human (rh)-rabphilin-3A or control lysate made from a 1:200 dilution of cell lysate was electrophoresed on a 7.5% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blotted with each patient's serum at a 1:50 dilution or with an anti-V5 antibody (1:1,000) as a positive control. A 76-kDa band was deemed positive if the band was detected in the rh-rabphilin-3A lysate lane but not in the control lysate lane.

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Table 1. Clinical Data of the 3 Patients With LINH Subjected to Shotgun LC-MS/MS Analysis of Immunoprecipitates

	Patient 1	Patient 2	Patient 3
Sex	Female	Female	Male
Age, y	32	31	19
Diagnosis	LINH	LINH	LINH
MRI findings	Swelling of the stalk	Swelling of the stalk	Swelling of the stalk
Biopsy	Yes	No	No
Anterior pituitary function	Normal	Normal	Normal
Posterior pituitary function	CDI	CDI	CDI

Preabsorption assay

Before use of the sera for Western blotting, each 25-μL serum sample was incubated for 1 hour at 4°C with 0.5 μg of bovine serum albumin (BSA) or a similar dose of rh-rabphilin-3A.

RNA extraction, RT-PCR, and quantitative real-time PCR

After decapitation, posterior pituitaries were removed from Sprague-Dawley rats (Chubu Science Materials) and used for RNA extraction. Total RNA was extracted and then reverse transcribed as described previously (19, 20). The primer sequences used are listed in Supplemental Table 1. The PCR product sizes of RPH3A, NLGN1, OTOF, USO1, SCG2, and RIMS1 were 360, 415, 487, 251, 376, and 493 bp, respectively. The PCR conditions were as follows: 35 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds, followed by a final extension at 72°C for 7 minutes.

Immunofluorescence

For immunofluorescence, cryostat sections (7 μm) were made of the pituitary gland from rats killed under deep ether anesthesia by transcardial perfusion with 4% paraformaldehyde in PBS, as described previously (19). Immunostaining was performed following a previously published procedure (19, 21). All fluorescently stained sections were examined with a fluorescence microscope (BZ-8000). The following antibodies were purchased: guinea pig polyclonal anti-vasopressin antibody (T-5048, 1:2000; Peninsula Laboratories), rabbit polyclonal anti-rabphilin-3A antibody (ab59259; Abcam), goat anti-guinea pig IgG-Alexa Fluor 488 (Invitrogen), and goat anti-rabbit IgG-Alexa Fluor 568.

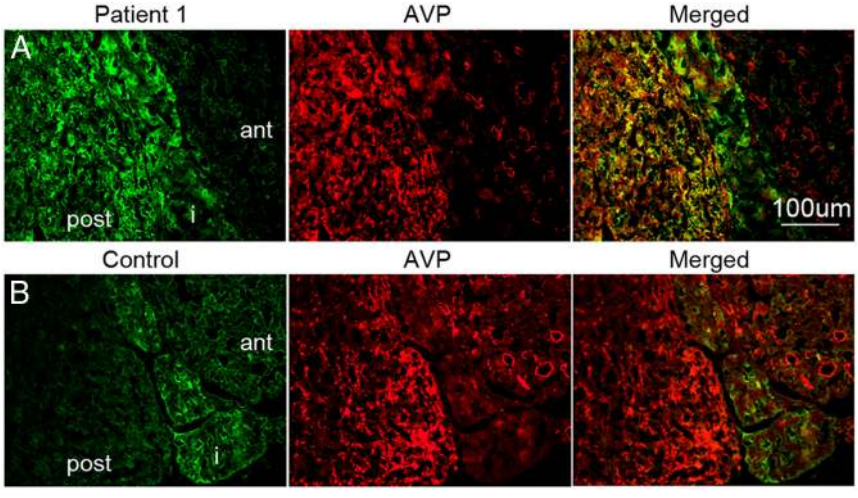

Figure 1. LINH patient serum recognizes the posterior pituitary gland. A and B, Representative images of double immunofluorescence of rat pituitary substrates that show the presence of autoantibodies against the posterior pituitary gland (A, left panel) in LINH patient serum but not in healthy control serum (B, left panel). The posterior pituitary gland is labeled with AVP (center panels). post, posterior lobe; i, intermediate lobe; ant, anterior lobe.

Results

LINH patient serum recognizes the posterior pituitary gland.

First, we used serum from 3 patients with LINH (1 was biopsy-proven LINH and the other 2 with convincing LINH as diagnosed clinically) (Table 1) as the case samples and healthy control serum samples to test for the presence of autoantibodies against the posterior pituitary gland. The sera from all 3 patients recognized the rat posterior pituitary gland (Figure 1A and Supplemental Figure 1, A and B), whereas the sera from 5 healthy subjects did not (Figure 1B). This result indicated the presence of autoantigens in the posterior pituitary gland and autoantibodies targeting the posterior pituitary gland in LINH patient sera.

Mass spectrometric analysis reveals six vesicle-associated proteins as candidates for autoantigens in patients with LINH

Because the patients with LINH showed autoantibodies against the posterior pituitary, we next sought to identify the target antigens exhaustively using mass spectrometry analysis after immunoprecipitation of posterior pituitary lysate with each serum sample. We identified 17 candidate proteins that overlapped in 3 patients and 12 in more than 2 patients but not in the healthy control subject (Figure 2). Among these candidates, we focused on vesicle-associated proteins because vasopressin is packaged into large, dense core vesicles, and vesicle-associated proteins are known to be targeted as autoantigens in several autoimmune diseases, including type 1 diabetes. Finally, we identified 6 candidates as autoantigens in LINH: RPH3A, NLGN1, OTOF, USO1, RIMS1, and SCG2 (Figure 2B). The mRNA expression of these 6 candidates was confirmed in hypothalamo-neurohypophysis (Supplemental Figure 2).

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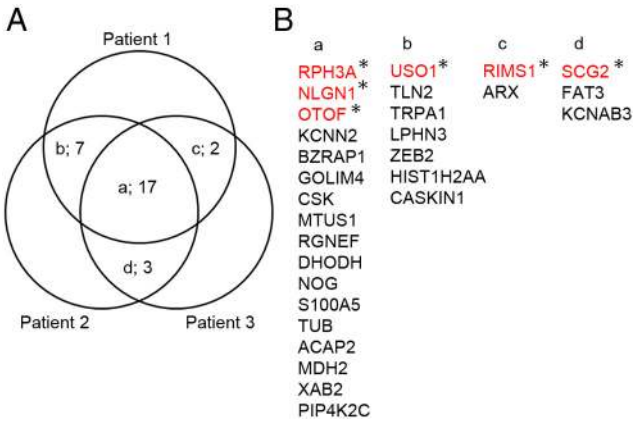

Figure 2. Candidate autoantigens among the 3 patients with LINH. A, Venn diagram showing the number of autoantigen candidates detected by mass spectrometry in each patient, as well as the candidates that overlapped between more than 1 patient. Listed gene names indicate the candidates identified in all 3 patients (a), in patient 1 and patient 2 (b), in patient 1 and patient 3 (c), and in patient 2 and patient 3 (d). B, Among the candidates, vesicle-associated proteins are marked with asterisk. RPH3A, rabphilin-3A; NLGN1, neuroligin-1; OTOF, otoferlin; KCNN2, small conductance calcium-activated potassium channel protein 2; BZRAP1; peripheral-type benzodiazepine receptor-associated protein 1; GOLIM4, Golgi integral membrane protein 4; CSK, tyrosine-protein kinase; MTUS1, mitochondrial tumor suppressor 1 homolog; RGNEF, ρ-guanidine nucleotide exchange factor; DHODH, dihydroorotate dehydrogenase mitochondrial; NOG, noggin; S100A5; protein S100–A5; TUB, tubby protein homolog; ACAP2, ARFGAP with coiled-coil, ANK repeat, and PH domain-containing protein 2; MDH2, malate dehydrogenase, mitochondrial; XAB2, pre-mRNA-splicing factor SYF1; PIP4K2C, phosphatidylinositol-5-phosphate 4-kinase type-2 γ; USO1, general vesicular transport factor p115; TLN2, talin-2; TRPA1, transient receptor cation channel subfamily A member 1; LPHN3, latrophilin-3; ZEB2, zinc finger E-box-binding homeobox 2; HIST1H2AA, histone H2A type 1-A; CASKIN1, caskin-1; RIMS1; regulating synaptic membrane exocytosis protein 1; ARX, homeobox protein ARX; SCG2, secretogranin II; FAT3, protocadherin Fat 3; KCNAB3, voltage-gated potassium channel subunit β-3.

LINH patient serum recognizes full-length recombinant human (rh) rabphilin-3A

To confirm recognition of the six candidate proteins by the patients' sera, we created full-length recombinant proteins using HEK293FT cells (Figure 3A). rh-rabphilin-3A was recognized by the serum of the 3 LINH patients but not by that of the healthy control subjects (Figure 3B). In contrast, we failed to detect recognition of the other 5 candidates by Western blot. To confirm whether this recognition was specific for rh-rabphilin-3A, we performed preabsorption studies. Preabsorption of the serum samples with rh-rabphilin-3A blocked the detection of the specific band using LINH patient serum (Figure 3C). In contrast, preabsorption of the serum samples with similar amounts of BSA did not diminish this band (Figure 3C), indicating that the LINH patient serum specifically recognized rabphilin-3A. We further performed immunohistochemical analysis to examine whether the patient serum recognized rh-rabphilin-3A in HEK293FT cells to test the influence of conformational structure under nonreducing conditions. The patient serum recognized rh-rabphilin-3A (Figure 3D and Supplemental Figure 3, A and B) but not untransfected HEK293FT cells (Figure 3G), whereas a disease control sample (Figure 3E) and a healthy control serum sample (Figure 3F) did not recognize rh-rabphilin-3A, suggesting that the epitopes targeted by the LINH autoantibodies were independent of the conformational structure of rabphilin-3A.

Rabphilin-3A is expressed in the posterior pituitary and AVP neurons in the hypothalamus

Because we showed that rabphilin-3A was a target of LINH autoantibodies, we next examined its expression in the posterior pituitary and the hypothalamic nuclei projecting to the posterior pituitary, which are inflamed in LINH. By immunofluorescence, we showed protein expression of rabphilin-3A in the rat posterior pituitary gland (Figure 4A) as well as in the supraoptic nucleus of the hypothalamus (Figure 4B), which was merged with AVP expression. In the human pituitary, rabphilin-3A expression was confirmed by Western blot (Figure 4C). These results suggest that rabphilin-3A expressed on AVP neurons is a target of LINH autoimmunity.

Clinical utility of the presence of anti-rabphilin-3A antibodies

Finally, we analyzed the clinical utility of the presence of anti-rabphilin-3A antibodies for the diagnosis of LINH. Specifically, we performed Western blotting to detect anti-rabphilin-3A antibodies in patients with biopsy-proven LINH (n = 4), clinically diagnosed LINH (n = 25), biopsy-proven LAH (n = 18), pathologically diagnosed sellar/suprasellar masses (n = 34), idiopathic CDI (n = 31), and other autoimmune diseases (n = 70) and in healthy control subjects (n = 41). In biopsy-proven diagnosis samples, the sensitivity for the presence of anti-rabphilin-3A antibodies was 100% (4 of 4) in patients with LINH (Figure 5A). The specificity was 96.2% (2 of 52) to differentially diagnose LINH from LAH or sellar/suprasellar masses (Figure 5A). In particular, the specificity was 100% for distinguishing sellar/suprasellar masses (0 of 34 including 18 patients with CDI) that were difficult to differentiate from LINH in clinical practice (Figure 5A). Ten of 70 samples from patients with other autoimmune diseases and 5 of 41 samples from healthy control subjects were also positive. Interestingly, 32.3% of idiopathic CDI samples also tested positive for anti-rabphilin-3A antibodies (Figure 5A), supporting the notion that LINH is a major cause of idiopathic CDI (7). The breakdown of diagnosis of the samples and percent positivity to anti-rabphilin-3A antibodies are shown in Supplemental Table 2. In fact, this marker was

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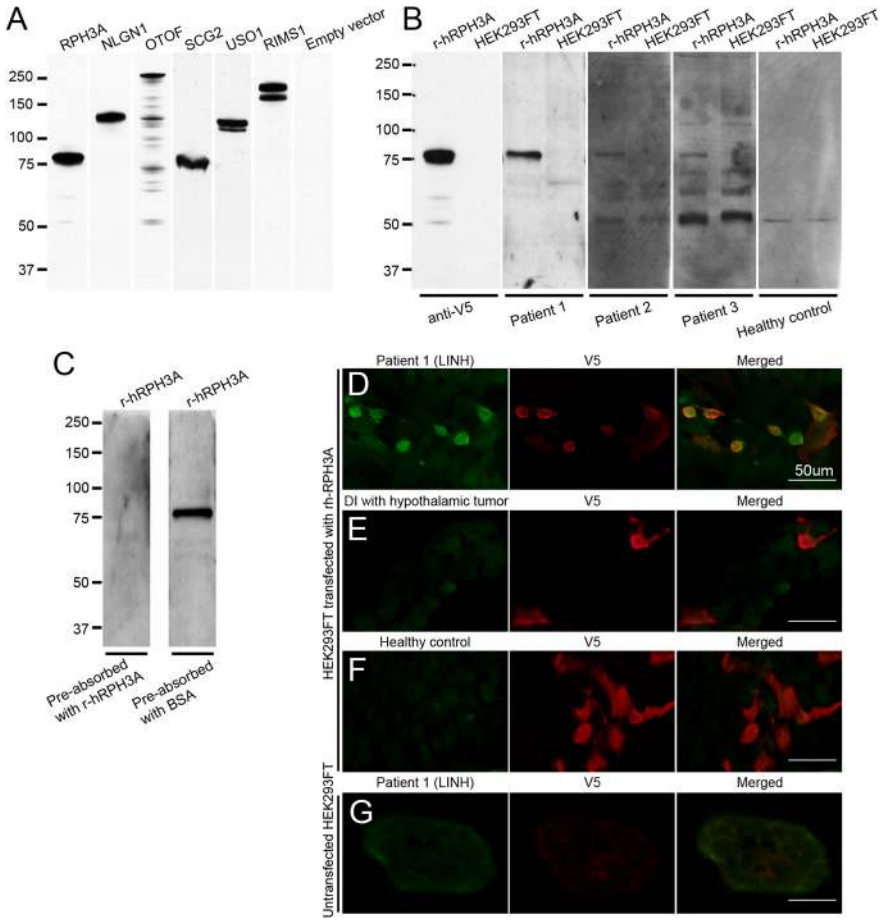

Figure 3. Western blot detection of anti-rabphilin-3A antibodies in LINH patient serum. A, Protein extracts from HEK293FT cells transfected with candidate genes were probed with an anti-V5 antibody. B, Recombinant full-length human rabphilin-3A (r-hRPH3A, left lanes) expressed in HEK293FT cells or total cell lysate from HEK293FT cells transfected with the empty vector (HEK293FT, right lanes) was probed with an anti-V5 antibody (positive control) or serum from LINH patient 1, 2, and 3 and a healthy control subject. C, Preabsorption experiment. r-hRPH3A was probed with patient serum preincubated with r-hRPH3A or BSA. D–G, Immunocytochemical detection of anti-rabphilin-3A antibodies in LINH patient serum. Double immunofluorescence shows colocalization of LINH patient 1 serum (green) and anti-V5 antibody (red) in HEK293FT cells transfected with r-hRPH3A (D). Serum from a patient with CDI due to a hypothalamic tumor (E) and that from a healthy control subject (F) did not recognize r-hRPH3A in HEK293FT cells. LINH patient serum did not recognize r-hRPH3A in untransfected HEK293FT cells (G).

useful to distinguish a patient with LINH from a patient with germinoma, who showed CDI and similar abnormal findings in the sellar and suprasellar areas on MRI scans (Figure 5, B and C). Overall, these results suggest that anti-rabphilin-3A antibodies could be useful to differentially diagnose LINH from sellar/suprasellar masses that can cause CDI.

Discussion

AH, including LINH, is often difficult to differentially diagnose from other pituitary diseases because of the similar presentation (eg, CDI) and radiographic appearance (sellar/suprasellar masses). Indeed, an invasive transsphenoidal pituitary biopsy is needed for a definite diagnosis. To identify a diagnostic marker, several autoantibodies, including anti-GH or anti-α-enolase, have been investigated for clinical utility. However, anti-α-enolase autoantibodies are not specific to pituitary autoimmune disease and have been reported in other autoimmune diseases. For example, anti-α-enolase antibodies were reported in 41.2%, 46.2%, and 23.5% of patients with lymphocytic hypophysitis, nonfunctional pituitary adenoma, and other pituitary diseases, respectively (10). With regard to LINH, there have been no reports of autoantigen candidates. In the present study, rabphilin-3A was predominantly recognized in the sera of patients with LINH (100% in patients with biopsy-proven LINH and 72% in patients with clinically diagnosed LINH). However, we detected some false-positives in healthy control samples (5 of 41), and given the high percentage of false-positives in other autoimmune diseases such as rheumatoid arthritis (14.2%), the healthy control samples we used might have included individuals with autoimmune diseases. Although we could not verify that 11% of patients with LAH were also positive for the autoantibodies, we assumed that the patients with LAH showing positivity for anti-rabphilin-3A antibodies may also have inflammation in the posterior pituitary gland, even though the patients presented with only anterior pituitary dysfunction without CDI. These possibilities may explain a few positive samples for anti-rabphilin-3A antibodies in patients with LAH (2 of 18). Furthermore, narrowing down the more specific epitopes of rabphilin-3A in patients with LINH might improve the specificity in future studies. In contrast, the strongest finding in this study was 100% specificity in patients with biopsy-proven sellar/suprasellar masses that were not lymphocytic hypophysitis. In addition, anti-rabphilin-3A antibodies were absent in all patients with CDI associated with sellar/suprasellar masses without lymphocytic hypophysitis. Importantly, the high specificity was confirmed in samples from both institutions in Japan and from Johns Hopkins University, demonstrating the usefulness of anti-rabphilin-3A antibodies as a diagnostic marker regardless of race.

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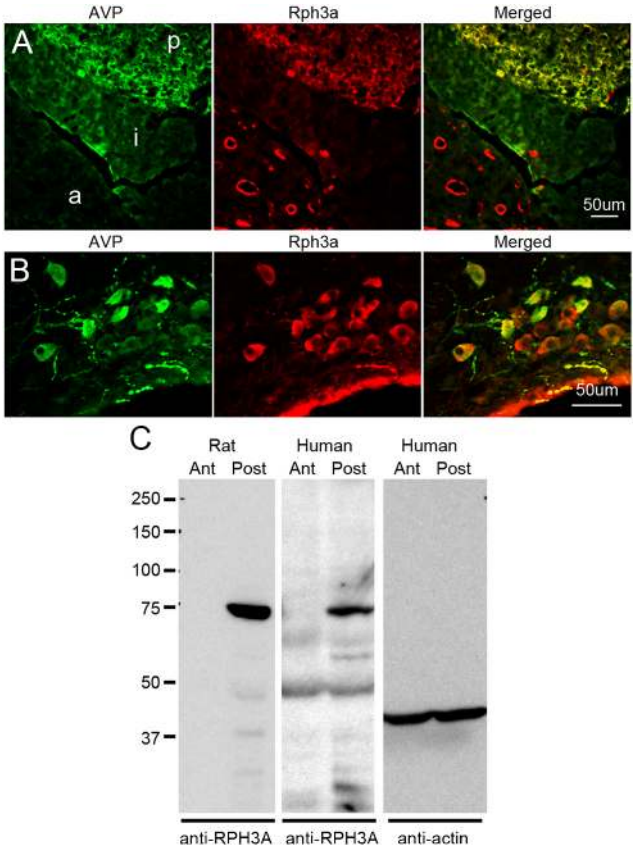

Figure 4. Rabphilin-3A expression in the posterior pituitary and supraoptic nucleus in rats and the human posterior pituitary. Double immunofluorescence shows the expression of rabphilin-3A in the posterior pituitary (A) and supraoptic nucleus (B) of rats. p, posterior lobe; i, intermediate lobe; a, anterior lobe. C, Protein extracts from the pituitary glands of rats and humans were probed with an anti-rabphilin-3A antibody. Ant, anterior lobe; post, posterior lobe.

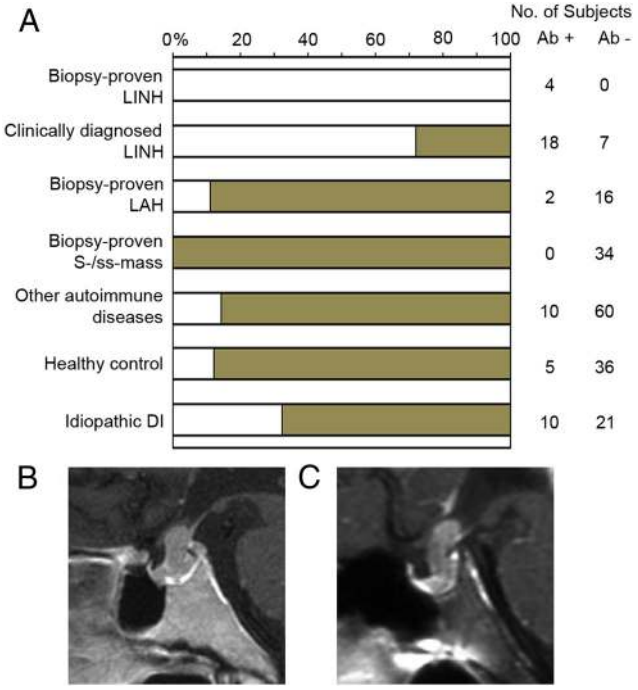

Figure 5. Summary of the presence of autoantibodies against rabphilin-3A. A, The white and dark bars indicate the presence and absence of autoantibodies against rabphilin-3A, respectively. The right 2 columns indicate the numbers of patients with and without autoantibodies to rabphilin-3A in each group. S-/ss-mass; sellar/suprasellar masses; Ab, autoantibodies. B and C, MRI scans enhanced with gadolinium showing enlargement of the posterior pituitary and stalk in a patient with biopsy-proven LINH (B) and a patient with biopsy-proven germinoma (C).

Imura et al (7) reported that 9 of 17 patients with idiopathic CDI showed stalk abnormalities with thickening and enlargement on MRI scans, which were observed only in patients who had CDI for less than 2 years. In addition, biopsies revealed chronic inflammation with lymphocytes in 2 patients. Our finding of a high prevalence of anti-rabphilin-3A antibodies in idiopathic CDI (32.3%) supports the notion that LINH is a major cause of idiopathic CDI. Maghnie et al (22) reported that among 36 patients with CDI whose pituitaries was normal size on MRI scans, 19 (53%) patients had a diagnosis of idiopathic CDI after follow-up for a median of 7.6 years (22) Although the collected idiopathic CDI sera contain samples from patients in whom CDI was diagnosed after long-time follow-up, it is possible that the sensitivity of anti-rabphilin-3A antibodies in LINH may be even higher in the future. In terms of pituitary function, Di Iorgi et al (1) reported that when patients with idiopathic CDI were stratified based on the pituitary stalk size at the time of diagnosis, among the 9 patients with normal pituitary stalk size at diagnosis, 5 developed a GH defect. Therefore, the probability of having a prognosis of survival of more than 10 years without development of any anterior pituitary hormone defects including a GH defect was 44.4%. We need to follow-up on patients in whom idiopathic CDI was diagnosed, and further studies are required to validate the use of anti-rabphilin-3A antibodies in patients with pituitary abnormalities, particularly those with idiopathic CDI and stalk thickening.

It has been reported that vasopressin-cell autoantibodies (AVPc-Abs) are detected in patients with idiopathic CDI or LINH (23–25). However, Maghnie et al (26) reported that AVPc-Abs were frequently present in patients with Langerhans cell histiocytosis and germinoma, indicating that AVPc-Abs cannot be considered a completely reliable marker of autoimmune CDI (26). These findings imply that there should be autoantigens in AVP neurons and that the identification of autoantigens in AVP neurons would improve the disease specificity of the autoantibodies. It would be interesting to evaluate the relationship between anti-rabphilin-3A antibodies and AVPc-Abs.

Rabphilin-3A, which was identified as an effector protein of Rab3A (27, 28), is expressed mainly in the brain. Rab3A belongs to the small G protein superfamily and is thought to be a critical regulator of secretory vesicle traf-

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ficking including exocytosis. Rabphilin-3A has been reported to regulate the release of neurotransmitters and hormones, and it is tempting to speculate on the role of anti-rabphilin-3A antibodies in LINH. Because we demonstrated expression of rabphilin-3A in the posterior pituitary gland and in AVP neurons in the hypothalamus, anti-rabphilin-3A antibodies may develop secondary to the onset of LINH, which causes destruction of the posterior pituitary gland. There are several autoantigens that have significance in disease pathogenesis in addition to serving as diagnostic markers, including thyroglobulin antibodies (29–31) and insulin antibodies (32, 33). Therefore, it remains to be determined whether rabphilin-3A is involved in the pathogenesis of LINH. T cells are thought to be critical for disease induction in hypophysitis based on findings from animal models in which the disease could be prevented by neonatal thymectomy and could not be transferred by passive transfer of autoantibodies (34, 35). However, the pathophysiologic role of anti-rabphilin-3A antibodies in this disease remains unclear.

In this study, we identified 5 other proteins known to function in vesicle exocytosis or trafficking as candidate autoantigens using LC-MS/MS analysis. Although we showed here that these 5 candidates were expressed at the mRNA level in hypothalamo-neurohypophysis, none were recognized by the 3 LINH patient serum samples by Western blot. Given that we only tested recognition under reducing conditions, it is possible that the patient sera may have recognized conformational epitopes in these candidates. Thus, a combination of the test for anti-rabphilin-3A antibodies by Western blot with another test for other candidates in their native conformations may provide a more specific diagnosis for LINH.

In conclusion, we report that rabphilin-3A is a predominant autoantigen in LINH. Therefore, clinical testing for anti-rabphilin-3A antibodies may be useful to differentially diagnose LINH from other pituitary diseases. These findings may lead to the development of diagnostic procedures that eliminate the need for invasive transsphenoidal pituitary biopsy.

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