**SUPPLEMENTARY MATERIAL**

**Argonaute autoantibodies as biomarkers in autoimmune neurological diseases**

**METHODS**

**Immunohistochemistry**

Freshly prepared adult rat brains were fixed in 4% paraformaldehyde (PFA) for 1 h, frozen, and sliced into 12 µm-thick sections. Immunolabeling was performed as previously described,e1 using patient CSF (1:10) and revealed with Alexa 488 fluorophore-conjugated secondary antibodies (1:1,200, A11013, Thermo Fischer, Courtaboeuf, France).

**Immunoprecipitation and MS-based proteomic analysis**

We used the CSF of a patient with LE and atypical staining by immunohistochemistry (patient XI), as well as a control (without staining pattern), to identify the target by immunoprecipitation and MS. A volume of 5 µL of CSF was mixed with 50 µL of protein G–conjugated agarose beads (Sigma Aldrich, Lyon, France) and adjusted to 500 µL with phosphate buffered saline (PBS). The mixture was incubated for 2 h at 4 °C with rotation to allow for the binding of CSF antibodies to the protein G. Simultaneously, a whole protein extract from one rat brain was prepared and incubated with 50 µL of agarose beads. Nonspecific contaminants were removed from the lysate by a 5 minute centrifugation at 16,000 g and 4 °C. The cleared lysate was subsequently used for immunoprecipitation with the antibody-conjugated agarose beads. The immunoprecipitate was analyzed by SDS-PAGE, followed by silver-staining, western blotting, and MS-based proteomics, as previously described.e2 Briefly, proteins were in-gel digested using modified trypsin (sequencing grade, Promega Corporation, Madison, WI, USA), and resulting peptides were analyzed by online nanoLC-MS/MS (UltiMate 3000 and Q-Exactive Plus, Thermo Fisher Scientific, Bremen, Germany) using a 75-min gradient. Peptides and proteins from different samples were identified using Mascot (version 2.6.0, Matrix Science, London, United Kingdom) through concomitant searches against the Uniprot database (*Rattus norvegicus* and *Homo sapiens* AD169 taxonomies, October 2019 version), classical contaminant database and the corresponding reversed databases. The Proline software,e3 was used to filter the results and compile, group, and compare the protein groups identified in each sample.

**Protein microarrays**

Sera from 34 patients with peripheral neuropathies (12 with SNN, 22 with other peripheral neuropathies) and 9 healthy controls were prepared as previously described,e4 and then tested on HuProt™ 3.1™ human proteome microarrays (CDI Laboratories, Baltimore, MD, USA) and analyzed as previously reported.e5 Briefly, the arrays were blocked, and sera were incubated for 2 h at room temperature at a 1:1,000 dilution. After washing, bound IgG were revealed with Alexa546-labelled polyclonal goat anti-human IgG(H+L) antibodies (Invitrogen, Cat. A21089, Renfrew, UK) and stained in parallel with goat anti-GST-650 to reveal all the proteins (tagged with glutathione S-transferase GST). The slides were scanned with a Tecan LS400 microarray scanner (Tecan Systems, Theale, UK) and the images analyzed with GenePix Pro (Molecular Devices, San Jose, CA, USA). A standard normalization between subjects (z-score statistics) was used to compare the reactivities of each antigen among the samples. A z-score cutoff of 4 standard deviations above the controls and a specificity of 100% (i.e., absent in all controls) were applied to identify antigen candidates.

**Cell-based assay**

To screen a large cohort for AGO-Abs, samples were firstly tested on AGO1 and AGO2-CBA; positive samples were then assayed on AGO3 and AGO4-CBA. HEK293 cells were transfected with VP5-HA-AGO1, VP5-HA-AGO2, VP5-HA-AGO3, VP5-HA-AGO4, VP5-HA-trinucleotide repeat containing 6A (TNRC6A), VP5-HA-TNRC6B, or VP5-HA-TNRC6C plasmid (kindly gifted by Professor Gunter Meister, University of Regensburg, Germany) for a transient overexpression.e6 TNRC6s genes were cloned in the same plasmid as AGOs via FseI and AscI restriction sites. Fixed and permeabilized cells were then immunostained as previously describede1 with patient CSF (1:10) or serum (1:100), and then revealed with Alexa555 fluorochrome-conjugated secondary goat anti-human IgG antibodies (1:1,200, A21433, Invitrogen, Villebon sur Yvette, France). Photographs of stained cells were taken with fluorescent microscope Axio Imager Z1 (Carl Zeiss, Marly le Roi, France). For double immunolabeling, rabbit anti-AGO2 (1:1,000) or rabbit anti-hemagglutinin (anti-HA, 1:1,000, P1985, Fisher Scientific, Villebon sur Yvette, France) was added to dilute CSF and was revealed by Alexa488-conjugated secondary goat anti-rabbit IgG antibody (1:1,000, A11034, Invitrogen). Antibody titers were obtained by using serial dilutions of serum (if available) and CSF on HEK293 cells expressing AGO1-4. Patient IgG subtypes in serum and CSF (if available) were identified using AGO1 or AGO2-transfected HEK293 cells and secondary anti-human antibodies specific for IgG1 (1:1,000, MCA4774, Bio-Rad, Marnes-la-Coquette, France), IgG2 (1:500, 555873, BD Biosciences, Le Pont de Claix, France), IgG3 (1:1,000, 5247-9850, Bio-Rad), or IgG4 (1:500, 555881, BD Biosciences). Bound IgG was revealed by a goat anti-mouse IgG antibody coupled with Alexa555 (1:1,000, A21424, Invitrogen).

**AGO1- and AGO2-Abs immunoadsorption**

A total of 1.6 x 106 HEK293 cells were seeded in 6-well plates. Cells were transfected with AGO1 or AGO2 plasmids by using LTX (10573013, Fisher Scientific) following manufacturer’s instructions. After 24 h, cells were fixed with 4% PFA. Then, 1 mL of CSF (1:100) from patient XI was incubated in each well for 24 h until no further CBA signal was observed. Diluted CSFs were then used for immunohistochemistry on rat brain section as described above. A control experiment was performed in the same way on non-transfected HEK293 cells.

**Identification of the AGO2 binding region**

Four domains of AGO2 (F1, F2, F3, F4) were amplified by PCR using the VP5-AGO2 plasmid as a template. The list of primers corresponding to each domain is described in e-table 1. Four plasmids each coding for one AGO2 domain were obtained and transfected into HEK293 cells, and immunolabeling with all available patient samples was conducted as described in the CBA section.

**Supplementary references**

e1. Do LD, Gupton SL, Tanji K, et al. TRIM9 and TRIM67 Are New Targets in Paraneoplastic Cerebellar Degeneration. Cerebellum Lond Engl. 2019;18:245–254.

e2. Casabona MG, Vandenbrouck Y, Attree I, Couté Y. Proteomic characterization of Pseudomonas aeruginosa PAO1 inner membrane. Proteomics. 2013;13:2419–2423.

e3. Bouyssié D, Hesse A-M, Mouton-Barbosa E, et al. Proline: an efficient and user-friendly software suite for large-scale proteomics. Bioinforma Oxf Engl. 2020;36:3148–3155.

e4. Moritz CP, Tholance Y, Lassablière F, Camdessanché J-P, Antoine J-C. Reducing the risk of misdiagnosis of indirect ELISA by normalizing serum-specific background noise: The example of detecting anti-FGFR3 autoantibodies. J Immunol Methods. 2019;466:52–56.

e5. Moritz CP, Stoevesandt O, Tholance Y, Camdessanché J-P, Antoine J-C. Proper definition of the set of autoantibody-targeted antigens relies on appropriate reference group selection. New Biotechnol. 2021;60:168-172.

e6. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 2004;15:185–197.

**Figure e-1. Confirmation that the atypical staining pattern was solely caused by antibodies targeting AGO proteins using immunoadsorption.**

Diluted patient XI’s CSF (1:100) depleted for only AGO2-Abs (A) or both AGO1-Abs and AGO2-Abs (B) were used for immunostaining on rat brain sections. Only a combination of AGO1 and AGO2-Abs depletion, but not AGO2 alone, was able to abolish the immunostaining signal.

**e-table 1. List of primers used for AGO2 epitopes mapping**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Fragment | Domain’s name (aa position) | Template | Forward primer | Reverse primer |
| F1-2 | N-terminus-PAZ | VP5-AGO2 | TTGTGGCATGAGAATTCAGTGGATCCACTAGT | ATTCTCATGCCACAATGTTACAGACCTCCA |
| F3-4 | MID-PIWI | VP5-AGO2 | GCGGCCGCGGACAAAGATGTATTAAAAAATTAA | TTTGTCCGCGGCCGCTAGCGTAATC |
| F1 | N-terminus (1-234) | F1-2 | AGTTTGTTTGAGAATTCAGTGGATCC | ATTCTCAAACAAACTCGATTACTGGCTGTGC |
| F2 | PAZ (235-348) | F1-2 | GCGGCCGCTGTGAAGTTTTGGATTTTAAAAG | CTTCACAGCGGCCGCTAGCGTAATC |
| F3 | MID (349-516) | F3-4 | GCCTGCAGTGAGAATTCAGTGGATCCACTAGT | ATTCTCACTGCAGGCCCGCATACGT |
| F4 | PIWI (517-859) | F3-4 | GCGGCCGCCTGGTGGTGGTCATCCTGC | CCACCAGGCGGCCGCTAGCGTAATC |