Supplementary Online Content

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This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Supplemental methods

Detection of neuronal antibodies against known paraneoplastic or cell surface antigens

All serum and CSF samples (70 paired samples; 39 sera, and 5 CSF) were examined for the presence of onconeural (Hu, Yo, Ri, CRMP5, amphiphysin, Ma2, Tr), GAD, and neuropil antibodies (NMDAR, AMPAR, $GABA_BR$, CASPR2, LGI1, DPPX, mGluR1, and mGluR5) using immunohistochemistry on frozen sections of paraformaldehyde-perfused or post-fixed rat brain, as reported.[1,2] Samples showing tissue immunoreactivity were further examined with immunoblot (Euroimmun, Lübeck, Germany) or cell based assays using HEK293 cells transfected with the appropriate plasmids.[3] All samples were examined for GlyR and $GABA_AR$ antibodies using live cell-based assays with the α1 subunit of the GlyR (serum diluted from 1:80, CSF 1:5) and the α1/β3 subunits of the $GABA_AR$ (serum diluted from 1:40, CSF 1:5) as reported.[4,5]

Screening for new antibodies against surface antigens

All samples were examined for IgG and IgM antibodies to unknown cell surface antigens by immunohistochemistry on post-fixed rat brain and immunofluorescence on live cultured rat hippocampal neurons. These experiments and immunoprecipitation assays were done as described (see below).[3,6]

Immunohistochemistry on post-fixed rat brain

Female Wistar rats were euthanized and the brain was removed, sagittally sectioned, immersed in 4% paraformaldehyde at 4°C for 1 hour, cryoprotected with 40% sucrose for 24 hours, and snap frozen in chilled isopentane. Immunohistochemistry using a standard avidin-biotin

peroxidase method was applied using patients' serum (diluted 1:200) or CSF (1:5), followed by the appropriate biotinylated secondary goat anti-human IgG antibodies (at dilution 1:2000) (Vector Labs, Burlingame, CA, USA) or goat anti-human IgM (at dilution 1:1000) (Southern Biotechnology, Birmingham, AL, USA) and the Vectastain Elite ABC complex (Vector Labs, USA) for 40 min. The reaction was developed with 0.05% diaminobenzidine with 0.01% hydrogen peroxide in phosphate-buffered saline (PBS) with 0.5% Triton X-100.[2]

Immunofluorescence on live cultured rat hippocampal

Rat hippocampal neuronal cultures were prepared as reported.[7] Fourteen days live neurons grown on coverslips were treated for 1 hour at 37º C with patients' or control serum (final dilution 1:200) or CSF (1:5). After removing the media and extensive washing with PBS, neurons were fixed with 4% PFA, and incubated with anti-human IgG or IgM (diluted 1:1000) Alexa Fluor secondary antibody (Molecular Probes, OR). Results were photographed under a fluorescence microscope using Zeiss Axiovision software (Zeiss, Thornwood, NY).[6]

Immunoprecipitation assays

Immunoprecipitation experiments were done with cultures of rat hippocampal neurons grown in 100mm wells, and incubated at 37°C with patients' CSF or serum (diluted 1:100) for 1 hour. Neurons were then washed with PBS, lysed with buffer containing protease inhibitors (P8340; Sigma Labs, St. Louis, MO, USA), and centrifuged at 16,000 g for 20 minutes at 4°C. The supernatant was retained, incubated with protein A/G agarose beads (20423; Pierce, Rockford, IL) overnight at 4°C, and centrifuged. To immunoprecipitate IgM antibodies, the protein A/G beads were coated with an IgG goat antibody against human IgM (1020-01; Southern Biotech, Birmingham, AL, USA). The pellet was resuspended in Laemmli buffer,

boiled for 10 minutes, separated in a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the proteins visualized with EZBlue gel staining (G1041; Sigma Labs). Because the EZBlue gel staining did not identify specific protein bands, gels were cut into ten slices and sent for mass spectrometry to the Proteomics Core Facility at the University of Pennsylvania. Protein bands were trypsin digested and analyzed with a nano liquid chromatography (nano LC)/ nanospray/ linear ion trap (LTQ) mass spectrometer (Thermo Electron Corporation, San Jose, CA) as reported.[8] The Xcalibur software (Thermo Scientific, Waltham, MA) was utilized to acquire the raw data and Sequest program (ThermoFinnigan, San Jose, CA; version SRF v. 5) to match the results with the UniProtKB/Swiss-Prot protein sequence database. The Scaffold 3.3 program was used to analyze the files generated. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least three identified peptides. The specificity of the results was confirmed by western blot and specific primary antibodies: mouse monoclonal anti-HNK-1 (Sigma Aldrich, St. Louis, MO, USA) and anti-GluR2/3 (AMPAR subunits, Millipore, Billerica, MA, USA).

Analysis of IgM immunoreactivity in CSF of patients with OMS and lung cancer

To characterize the CSF IgM immunoreactivity of OMS patients on brain immunohistochemistry, a protein extract from human brain myelin prepared as described,[9] was separated in a sodium dodecyl sulfate polyacrylamide gel electrophoresis as described, transferred to immobilon-P membrane (Millipore IPVH00010) and incubated with an IgM monoclonal antibody against HNK-1 (Sigma-Aldrich, St. Louis, MO, USA), a serum positive for IgM MAG antibodies, and the three CSF from the OMS patients that showed the IgM immunoreactivity in brain immunohistochemistry. The reactivity was developed using the

appropriate biotinylated secondary antibodies (1:1000) and the avidin-biotin peroxidase and diaminobenzidine method.

To confirm that the IgM immunoreactivity of OMS patients colocalize with the HNK-1 epitope in vivo, live hippocampal neurons were incubated with the CSF showing the IgM immunoreactivity and the monoclonal antibody against HNK-1 and analyzed by immunoflurescence as described above. Colocalization analysis was done with Imaris 7.6 software (Bitplane AG, Zurich).

eReferences

1. Graus F, Dalmau J, Valldeoriola F, et al. Immunological characterization of a neuronal antibody (anti-Tr) associated with paraneoplastic cerebellar degeneration and Hodgkin's disease. J Neuroimmunol 1997;74:55-61.
2. Ances BM, Vitaliani R, Taylor RA, Liebeskind DS, Voloschin A, Houghton DJ, et al. Treatment-responsive limbic encephalitis identified by neuropil antibodies: MRI and PET correlates. Brain 2005;128:1764-77.
3. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol 2011;10:63-74.
4. Martinez-Hernandez E, Sepulveda M, Rostasy K, et al. Antibodies to aquaporin 4, myelin-oligodendrocyte glycoprotein, and the glycine receptor alpha1 subunit in patients with isolated optic neuritis. *JAMA Neurol* 2015;72:187-193.
5. Petit-Pedrol M, Armangue T, Peng X, et al. Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies. *Lancet Neurol* 2014;13:276-286.
6. Lancaster E, Lai M, Peng X, et al. Antibodies to the GABA(B) receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. Lancet Neurol. 2010;9:67-76.
7. Buchhalter JR, Dichter MA. Electrophysiological comparison of pyramidal and stellate nonpyramidal neurons in dissociated cell culture of rat hippocampus. Brain Res Bull 1991;26:333-8.
8. Strader MB, Tabb DL, Hervey WJ, Pan C, Hurst GB. Efficient and specific trypsin digestion of microgram to nanogram quantities of proteins in organic-aqueous solvent systems. Anal Chem. 2006;78:125-34.
9. Quarles RH, Pasnak CF. A rapid procedure for selectively isolating the major glycoprotein from purified rat brain myelin. Biochem J. 1977;163:635-7.
10. Luque FA, Furneaux HM, Ferziger R, et al. Anti-Ri: an antibody associated with paraneoplastic opsoclonus and breast cancer. Ann Neurol 1991;29:241-251.
11. Hersh B, Dalmau J, Dangond F, Gultekin S, Geller E, Wen PY. Paraneoplastic opsoclonus-myoclonus associated with anti-Hu antibody. Neurology 1994;44:1754-5.
12. Arino H, Hoftberger R, Gresa-Arribas N, et al. Paraneoplastic Neurological Syndromes and Glutamic Acid Decarboxylase Antibodies. JAMA Neurol 2015;72:874-881.
13. Markakis I, Alexiou E, Xifaras M, Gekas G, Rombos A. Opsoclonus-myoclonus-ataxia syndrome with autoantibodies to glutamic acid decarboxylase. Clin Neurol Neurosurg 2008;110:619-621.
14. Bhandari HS. Presentation of opsoclonus myoclonus ataxia syndrome with glutamic acid decarboxylase antibodies. BMJ Case Rep 2012;2012.
15. Bataller L, Rosenfeld MR, Graus F, Vilchez JJ, Cheung NK, Dalmau J. Autoantigen diversity in the opsoclonus-myoclonus syndrome. *Ann Neurol* 2003;53:347-353.

16. Candler PM, Dale RC, Griffin S, et al. Post-streptococcal opsoclonus-myoclonus syndrome associated with anti-neuroleukin antibodies. J Neurol Neurosurg Psychiatry 2006;77:507-512.
17. Zaro-Weber O, Galldiks N, Dohmen C, Fink GR, Nowak DA. Ocular flutter, generalized myoclonus, and trunk ataxia associated with anti-GQ1b antibodies. Arch Neurol 2008;65:659-661.
18. Morales-Briceno H, Rodriguez-Violante M, Cervantes-Arriaga A, Irani SR, Lees AJ, Silveira-Moriyama L. Opsoclonus myoclonus syndrome associated with GQ1b antibodies. Mov Disord 2012;27:1615-1616.
19. Smith JH, Dhamija R, Moseley BD, et al. N-methyl-D-aspartate receptor autoimmune encephalitis presenting with opsoclonus-myoclonus: treatment response to plasmapheresis. Arch Neurol 2011;68:1069-1072.
20. Kurian M, Lalive PH, Dalmau JO, Horvath J. Opsoclonus-myoclonus syndrome in anti-N-methyl-D-aspartate receptor encephalitis. Arch Neurol 2010;67:118-121.
21. DeFelipe-Mimbrera A, Masjuan J, Corral I, Villar LM, Graus F, Garcia-Barragan N. Opsoclonus-myoclonus syndrome and limbic encephalitis associated with GABAB receptor antibodies in CSF. J Neuroimmunol 2014;272:91-93.

eTable 1. Univariate analysis for factors associated with bad outcome (mRS >2) at last follow up

Factor	p	OR	95% CI
Age Over 40	0.002*	6.8	2.1-22.2
Gender	0.219	1.8	0.7-4.4
Paraneoplastic	<0.001*	7.9	2.8-22.3
Tumor Type	<0.001*		
No Tumor	Reference		
Lung cancer	<0.001	16.7	4.2-66.6
Breast cancer	0.027	6.9	1.3-37.6
Teratoma	0.179	3.1	0.6-16.0
Other tumor	0.660	1.7	0.2-19.0
Prodromal Symptoms	0.361	0.6	0.7-1.9
Encephalopathy[+]	0.062*	2.7	1.0-7.4
Abnormal craneal nerves	0.232	2.4	0.6-9.5
Onconeuronal antibodies	0.008*	18.2	2.1-154.3
Neuronal surface antibodies[&]	0.048*	3.2	1.0-10.2
Immunotherapy	0.247	2.6	0.5-13.2
Specific Treatment	0.017*		
Not treated	Reference		
Steroids	0.436	2	0.3-11.4
IVIG	0.538	0.4	0.03-5.9
Steroids+IVIG	0.197	3.1	0.6-17.9
Steroids and/or IVIG+PEX	0.019	14	1.5-127.2
Relapse	0.029*	4.3	1.7-15.7
Months Follow up (log_e)[$]	<0.001*	0.4	0.3-0.6

CI = confidence interval; IVIg= intravenous immunoglobulin; log_e = natural logarithm; OR = odds ratio; PEX= plasma exchange; mRS = modified Rankin Scale.

[*]Analysis only included patients with follow-up >3months (N=81). Factors associated with a bad outcome ($p<0.1$) were included in a multivariable binary logistic regression model and approached by forward stepwise procedure.

[+]Includes decreased level of consciousness and/or severe abnormal behavior. Minor abnormal behavior changes as mild anxiety or insomnia were not included.

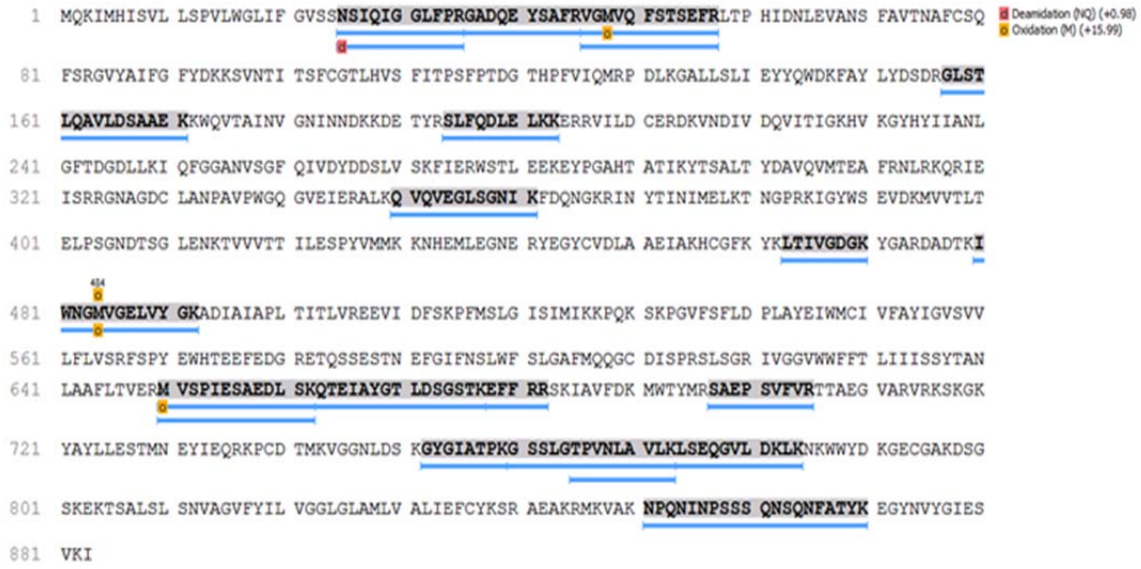
[&]Includes IgM (HNK1) and IgG (Glycine, NMDAR, $GABA_BR$, DPPX or unknown antigens) antibodies.

[$]Because of a skewed distribution, log-transformation was used for time of follow up (months).

eTable 2. Antibodies reported in adult patients with OMS

Antigen[(ref)]	Type of OMS	Cancer	Comment
Onconeural			
Ri (NOVA)[10]	Paraneoplastic	Breast	OMS usually in the setting of a wider brainstem encephalitis. Ri good biomarker of breast and gynecologic cancer
HuD, CRMP5, amphiphysin, SOX1, Zic4[11]	Paraneoplastic	Lung	A few cases reported, present series
Ma2	Paraneoplastic	NSCLC, gastric	Present series
Synaptic intracellular			
GAD[12-14]	Idiopathic/ paraneoplastic	SCLC	A few cases reported, present series concurrent with HNK1
Adenomatous poliposis coli (APC)[15]	Idiopathic		Two cases reported. Antibodies also found in two PNS patients without OMS
Surface antigens			
Neuroleukin[16]	Idiopathic		Two teenager cases after streptococcal infections
GQ1b[17,18]	Idiopathic		IgG and IgM antibodies
NMDAR[19,20]	Idiopathic/paraneoplastic	Ovarian teratoma	Present series, In two previous cases, opsoclonus was the presenting symptom of NMDAR encephalitis
$GABA_AR$[5]	Idiopathic		Two cases, antibody present only in serum
$GABA_BR$[21]	Idiopathic		Single case, patient developed limbic encephalitis

eFigure. Peptide sequences corresponding to GluR2 of AMPA receptor immunoprecipitated by the CSF of patients with IgM immunoreactivity against NHK-1



Peptide	Uniq	-10lgP	Mass	ppm	m/z	z	RT	Scan	#Spec	Start	End	PTM
K.QTEIAYGTLDSGSTK.E	N	60.43	1569.7522	-106	785.8	2	8.35	656	2	663	677	
K.IWNGM(+15.99)VGELVYGK.A	N	57.15	1480.7384	331.8	741.6224	2	14.2	1731	2	480	492	(M)
R.VGM(+15.99)VQFSTSEFR.L	N	55.58	1402.655	530.8	702.7076	2	10.37	1038	1	46	57	(M)
K.NPQNINPSSSQNSQNFATYK.E	N	54.9	2238.03	182.6	1120.2268	2	7.95	574	2	851	870	
R.GLSTLQAVLDSAAEK.K	N	54.39	1501.7987	423.6	752.2251	2	20.79	2964	1	157	171	
R.M(+15.99)VSPIESAEDLSK.Q	N	54.34	1420.6755	312.4	711.5673	2	9.49	878	4	650	662	Oxidation
K.GSSLGTPVNLAVLK.L	Y	53.15	1354.782	-505.5	678.0553	2	14.71	1823	4	760	773	
R.VGMVQFSTSEFR.L	N	52.58	1386.6602	383.5	694.6036	2	13.89	1675	2	46	57	
G.TPVNLAVLK.L	N	51.52	953.5909	427.7	478.0071	2	10.87	1129	3	765	773	
K.QVQVEGLSGNIK.F	N	51.15	1270.6881	395.2	636.6028	2	8.64	716	2	350	361	
S.NSIQIGGLFPR.G	N	50.88	1200.6615	-482	601.0482	2	16.08	2076	4	25	35	
R.SAEPSVFVR.T	N	48.99	990.5134	552	496.5379	2	8.81	751	4	697	705	
R.MVSPIESAEDLSK.Q	N	48.78	1404.6807	135.8	703.4431	2	11.88	1312	2	650	662	
R.GADQEYSAFR.V	N	48.66	1142.4993	294.8	572.4256	2	8.16	619	1	36	45	
K.GYGIATPK.G	N	46.73	805.4333	1126.7	404.1788	2	7.88	559	3	752	759	
K.LSEQGVLDKLK.N	N	41.36	1228.7026	188.1	615.4743	2	9.52	884	2	774	784	
K.LTIVGDGK.Y	N	38.89	801.4596	678.1	402.0095	2	8.06	597	3	463	470	
S.N(+.98)IQIGGLFPR.G	N	35.7	1201.6455	855.2	602.3447	2	18.43	2515	1	25	35	(NQ)
R.SLFQDLELKK.E	N	28.04	1219.6812	-251.1	407.4653	3	13.69	1637	1	194	203	
K.EFFRR.S	N	16.3	753.3922	620.8	377.9378	2	7.87	556	1	678	682	
total 20 peptides												