Supplementary Appendix

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

Supplement to: Nieuwkamp DJ, Murk J-L, van Oosten BW, et al. PML in a patient without severe lymphocytopenia receiving dimethyl fumarate. N Engl J Med 2015;372:1474-6. DOI: 10.1056/NEJMc1413724

Supplementary Materials for

PML in a Patient on Compounded Dimethyl Fumarate without Severe Lymphocytopenia

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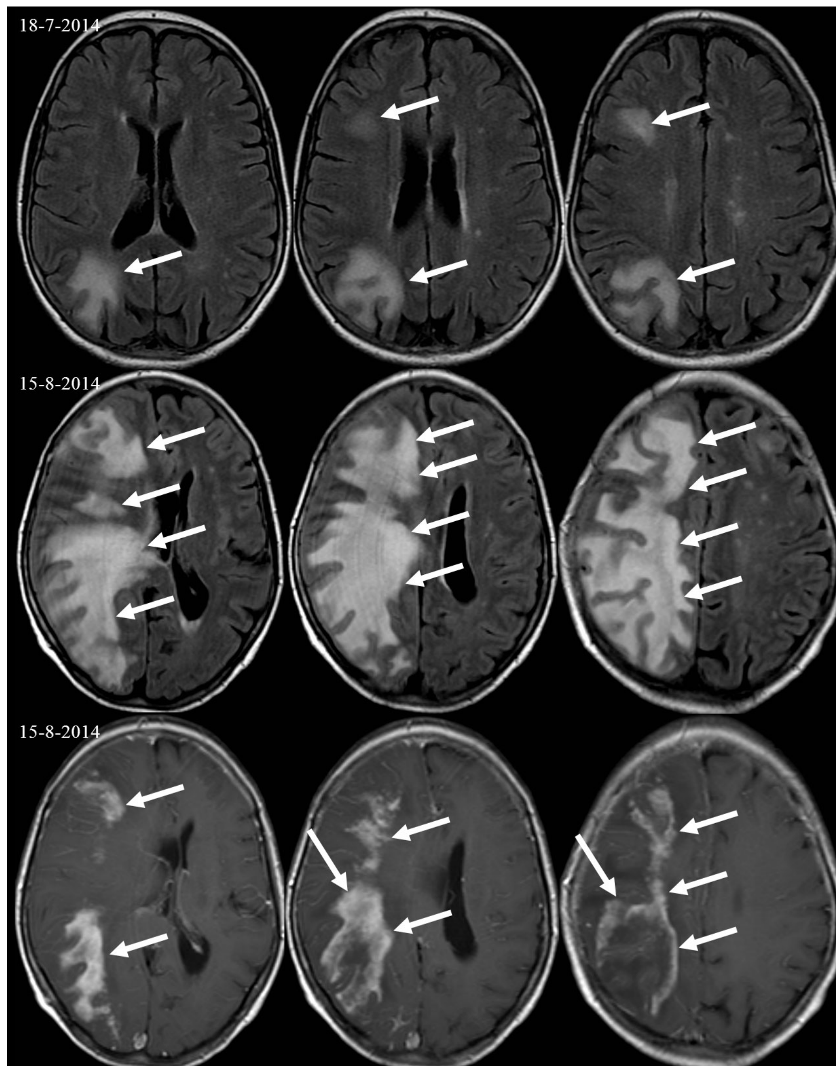
Supplementary Figures

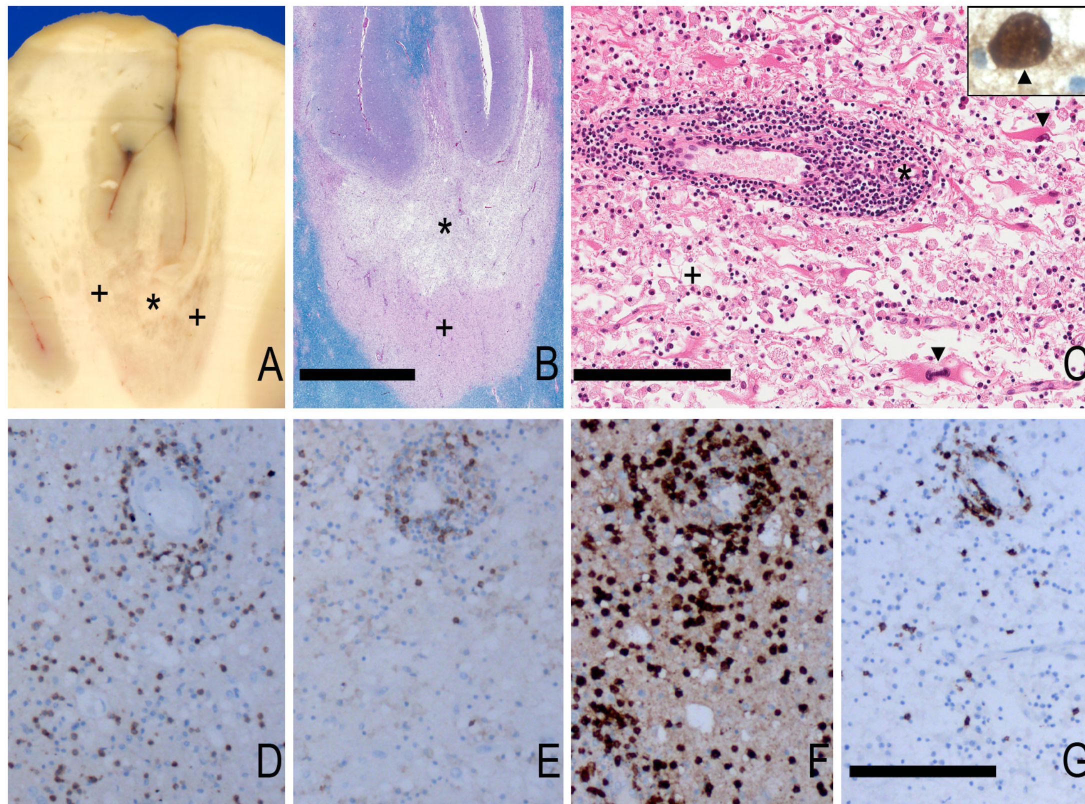
Figure S1. Axial MRIs of the brain showing manifestations of PML and signs of IRIS



Axial FLAIR (top and middle row) and contrast-enhanced T1-weighted images (bottom row) illustrating the PML-IRIS lesions evolution over one month time. The multifocal PML lesions (arrows top row) on the initial MRI demonstrated a rapid progression with confluent widespread lesion dissemination (arrows middle row), impressive signs of inflammation with contrast-enhancement (arrows bottom row) and mass effect leading to (subfalcine) herniation.

PML, progressive multifocal leukoencephalopathy; IRIS, immune reconstitution inflammatory syndrome

Figure S2. PML histopathology



A. Multiple foci of gray discoloration in the white matter (plus signs). There is cavitation of the subcortical white matter beneath the central adjacent sulci (asterisk).

B. Section stained for myelin with luxol and periodic acid schiff showing extensive loss of myelin (plus sign) and cavitation (asterisk). Scale bar= 6 mm

C. Focus of demyelination showing atypical astrocytes with very large nuclei (arrowheads), foamy macrophages (plus sign) and a perivascular cuff of lymphocytes (asterisk). Scale bar = 200 μm. Inset shows magnification of diaminobenzidine (DAB) stained swollen nucleus (arrowhead) of oligodendrocyte infected with JCV. Immunohistochemistry was performed with antibody to SV40 T-antigen.

D-G. Immunohistochemical stainings showing the perivascular cuff and intraparenchymal infiltration of T-lymphocytes (D: CD3 cells, E: CD4 cells, F: CD8 cells) and B-lymphocytes (G: CD20 cells), indicating a fulminant immune response. Scale bar for D-G= 200 μm

PML, progressive multifocal leukoencephalopathy

Supplementary methods

Results of virologic analyses

JCV-PCR was positive after autopsy on brain tissue and CSF. No BK virus or herpesvirus was detected. Sequencing of whole JCV genome revealed the patient was infected with genotype 1a. JCV in brain tissue and CSF showed a 38 nucleotide deletion in the non-coding control region (NCCR). Alterations of NCCR are typical for JCV in PML lesions. A blood sample was also JCV positive and contained both the archetype JCV and the NCCR deletion mutated JCV found in the brain.

Methods

Viral DNA Extraction and real-time Taqman PCR

Viral DNA was isolated from patient material using a MagNaPure 96 automated extraction system (Roche, Penzburg, Germany). Phocine Herpes Virus was added to the material prior to DNA extraction as an internal control. Samples were assayed in a 25 µl reaction mixture containing 10 µl isolate, Taqman universal PCR mastermix (applied Biosystems, ABI), primers (300nM diagnostic primers) and fluorogenic probe (200nM diagnostic probe) labelled with `5 reporter dye (FAM) and `3 quencher dye (TAMRA). See table S1-A for the primers and probe sequences of JCV real-time PCR. The amplification and detection were performed with an ABI 7500 system for 2 minutes at 50ºC, 10 minutes at 95ºC, and 45 cycles of 15 seconds at 95ºC and 1 minute at 60ºC. Samples were controlled for the presence of possible inhibitors of the amplification reaction by the indicated internal control, of which the signals had to be within the reference range.

Sequencing of JCV DNA

The whole genome sequence of JCV was obtained with the use of a modified sequence method described by Tan et al.[1] JCV PCR fragments were obtained by fractional amplification of MagNAPure 96 total DNA isolates using the Superscript III one-step Reverse Transcriptase-PCR System with Platinum Taq High Fidelity kit (invitrogen) and a 9800 Fast thermal cycler (ABI) according to the manufacturers protocol, but without reverse transcriptase step, (see table S1-B for the sequences of primers). PCR products were applied to a 1% agarose gel and purified from the gel with the use of GeneJet PCR purification kit (ThermoScientific). Isolated fragments were used for whole genome sequencing (see table S1-C for sequencing primers). Sequencing was performed by Macrogen Europe (Amsterdam, the Netherlands). The resulting sequence information was assembled into JC virus whole genome sequences through alignment with the reference JC polyomavirus [NC_001699.1] using Seqman Pro software (Lasergene 11 software, DNASTART, INC.).

Reference

1. Tan L, Lemey P, Houspie L, et al. Genetic Variability among Complete Human Respiratory Syncytial Virus Subgroup A Genomes: Bridging Molecular Evolutionary Dynamics and Epidemiology. PLoS One 2012;7(12):e51439. doi: 10.1371/journal.pone.0051439. Epub 20.

Supplementary Table

Table S1. Primers and probe used for JCV real-time PCR, whole genome amplification and sequencing

A. Diagnostic Real-time PCR

Name:	**5’-3’ sequence:**
Large T forward primer	CCACCCCAGCCATATATTGC
Large T reverse primer	TTGAAAGGTTTAATTTYTTGCTTGATAA
probe	FAM- AAACAGCATTGCCATGTGCCCCA-TAMRA

B. General primers for JC virus whole genome amplification

Name:	**5’-3’ sequence:**	*** Start:**	*** End:**
JC F1 forward primer	GCAGTCAACCATATGCCTTG **ATGGTTCTTCGCCAGCTG**	277	294
JC F1 reverse primer	GCTTGCGGAATTCTGACAC **CTATTGCCCAAGTAGTTTCTTCC**	1116	1138
JC F2 forward primer	GCAGTCAACCATATGCCTTG **CAACAGTTGGGCTTTTTCAG**	854	873
JC F2 reverse primer	GCTTGCGGAATTCTGACAC **CACACTGTAACAAGGAAGCATG**	1696	1717
JC F3 forward primer	GCAGTCAACCATATGCCTTG **GGGGTTGACTCAATTACAGAG**	1565	1585
JC F3 reverse primer	GCTTGCGGAATTCTGACAC **CCTCAAAAACTCTAACCTCCTC**	2432	2453
JC F4 forward primer	GCAGTCAACCATATGCCTTG **CTACCCAATTTCTTTCCTTCTTAC**	2341	2364
JC F4 reverse primer	GCTTGCGGAATTCTGACAC **GGATTTACCTTCAGGGCATG**	3199	3218
JC F5 forward primer	GCAGTCAACCATATGCCTTG **CAGAGCAGCTTAGTGATTTTCTC**	2986	3008
JC F5 reverse primer	GCTTGCGGAATTCTGACAC **GCTTATGGGCATGTACTTAGACT**	3784	3806
JC F6 forward primer	GCAGTCAACCATATGCCTTG **CTTTGTTTGGCTGCTACAGTATC**	3621	3643
JC F6 reverse primer	GCTTGCGGAATTCTGACAC **AATGGTTTGGGTGTGACTTAAC**	4562	4583
JC F7 forward primer	GCAGTCAACCATATGCCTTG **GTATTCCACCAGGATTCCCA**	4383	4402
JC F7 reverse primer	GCTTGCGGAATTCTGACAC **CGCCTCCGCCTCCA**	5117	5130
JC F8 forward primer	GCAGTCAACCATATGCCTTG **CAGGCTGATGAGCAACTTTTAC**	4797	4818

JC F8 reverse primer	GCTTGCGGAATTCTGACAC **CAGAAGCCTTACGTGACAGC**	291 310
C. Sequence primers for whole genome PCR fragments		
Name:	**5'-3' sequence:**	
Sequence forward primer	GCAGTCAACCATATGCCTG	
Sequence reverse primer	GCTTGCGGAATTCTGACAAC	

* Nucleotide position relative to reference JC polyomavirus [NC_001699.1]

A. Sequences of JCV primers and probe for real-time PCR assay to test clinical samples for presence of JCV.

B. Sequences of JCV forward and reverse primers used for the fractional amplification of JCV DNA in clinical samples. JCV specific sequences are depicted in bold. With these primers the whole JCV genome may be amplified. Each primer also contains 20 nucleotides that function as scaffold for sequencing primers.

C. Sequences of sequencing primers used for sequencing JCV PCR genome fragments