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Supplementary Materials for

Prion seeding activity and infectivity in skin samples from patients with sporadic Creutzfeldt-Jakob disease

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Methods

Study oversight

The use of human brain tissues was authorized by the Institutional Review Board, which is recognized by the Office for Human Research Protections of the U.S. Department of Health and Human Services. The Institutional Animal Use and Care Committee and the Institutional Biosafety Committee approved all of the animal experiments used in this study. The analyses of human specimens that were performed at the National Institute of Allergy and Infectious Diseases were performed under Exemption 13244 for the use of encoded samples from the National Institutes of Health Office of Human Subjects Research Protections.

Skin tissue preparation

After being washed three times in 1 X PBS and chopped into small pieces, the skin homogenates at 10% (w/v) were prepared in TBS containing 2 mM $CaCl_2$ and 0.25% (w/v) collagenase A (Roche) and incubated in a shaker at 37°C for 4 h. After sonication to disrupt remaining tissue structures, the samples were centrifuged for 5 min at 500 g. While the pellet (P1) was discarded, the supernatant (S1) was further centrifuged for 50 min at 85,000 g at 4°C to obtain the supernatant (S2) and the pellet (P2). The supernatant (S2) was then discarded, and the pellet (P2) was re-suspended in 1 ml 1% Sarcosyl in TBS. The suspension was centrifuged for 2.5 h at 150,000 g. The next supernatant (S3) was discarded and the pellet (P3) was re-suspended in 1 ml 0.1% Sarcosyl, 10% (w/v) NaCl in TBS, with PK added to a final concentration of 25 µg/ml. After incubating at 37°C for 30 min, 5 µl of protease inhibitor cocktail (Sigma) was added into the sample to inhibit PK according to the product instructions. The samples were then subjected to a final centrifugation at 150,000 g for 2.5 h at 4°C and the supernatant (S4) was discarded. The pellet (P4) containing the CJD-specific PrP^{Sc} was re-suspended in 50 µl of PBS for RT-

QuIC analysis. For Western blot analysis, 20 µl of SDS sample buffer was added and heated at 100°C for 5 min, prior to Western blotting to detect PK-treated PrP^{Sc}. For treatment with PK and PNGase F, the PK-treated samples were further deglycosylated using PNGase F (New England Biolabs), in accordance with the manufacturer's instructions, followed by Western blotting. In addition to protein chemistry analysis, skin samples from two vCJD, two sCJD and two non-CJD patients were also obtained for fixation in 10% formalin for further histological analysis (see below).

Molecular genetic analysis

Genomic DNA was extracted from skin samples as described above and a 760 bp fragment corresponding to the human PrP gene (residues 5 to 258) was PCR amplified using primers HRM-F (5'-TATGTGGACTGATGTCGGCCTCTGCAAGAAGCGC-3') and HRM-R (5'-CCACCTCAATTGAAAGGGCTGCAGGTGGATAC-3') with defined cycling conditions (*15, 38*). The Met/Val polymorphism at codon 129 and mutation of the PrP gene coding region were determined as previously described (*15*) or by Sanger and deep sequencing (*38*). Nucleotide sequences from both deep and Sanger sequencing were analyzed using DNASTAR Lasergene Software Suite v.7.1.0 (Madison, WI).

RT-QuIC analysis

The RT-QuIC analysis of skin or brain samples was conducted as described previously (*14, 21*). In brief, RT-QuIC reaction mix was composed of 10 mM phosphate buffer (pH 7.4), 300 or 130 mM NaCl, 0.1 mg/ml recombinant bank vole (BV; residues 23-230, M109) or Syrian golden hamster (Ha; residues 23-231) $rPrP^{Sen}$, 10 µM Thioflavin T (ThT), 1 mM ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA), and 0.002% or 0.001% SDS.

NaCl and SDS concentrations were varied as needed. Aliquots of the reaction mix (98 µl) were loaded into each well of a black 96-well plate with a clear bottom (Nunc) and seeded with 2 µl of skin homogenate or brain homogenate dilutions. Both the S1 and P4 skin fractions were spun at 2,000 g for 2 min at room temperature prior to making serial dilutions. The plate was then sealed with a plate sealer film (Nalgene Nunc International) and incubated at 42°C in a BMG FLUOstar Omega plate reader with cycles of 1 min shaking (700 rpm double orbital) and 1 min rest throughout the indicated incubation time. ThT fluorescence measurements (450 +/-10 nm excitation and 480 +/-10 nm emission; bottom read) were taken every 45 min. Four replicate reactions were seeded with the same dilution of an individual sample. The average fluorescence values per sample were calculated using fluorescence values from all four replicate wells regardless of whether these values crossed the threshold described below. To compensate for minor differences in baselines between fluorescent plate readers and across multiple experiments, data sets were normalized to a percentage of the maximal fluorescence response (260,000 rfu) of the plate readers after subtraction of the baseline, as described (*14*, *21*), and plotted versus reaction time. Reactions were classified as RT-QuIC positive based on criteria similar to those previously described for RT-QuIC analysis of brain specimens (*21*). Briefly, a ThT fluorescence threshold for a reaction to be considered positive was based on the mean ThT value of all negative control samples at 50 hours, plus 20 standard deviations (*26*) yielding an ~8.8% threshold. At least 2 of 4 replicate wells must cross this threshold for a sample to be considered positive.

Construction of transgenes expressing human PrP-129V or PrP-129M

The HuPrP-129V or-129M open reading frame (ORF) was amplified from the human genomic DNAPAC (P1-derived artificial chromosome) clone RP5–1068H6 (obtained from the Sanger Center, Cambridge, UK) with primers HRM-F (TATGTGGACTGATGTCGGCCTCTGCAAGAAGCGC) and HRM-R (CCACCTCAATTGAAAGGGCTGCAGGTGGATAC) (*24*, *25*). The PCR product was digested with PshAI and MfeI and used to replace the corresponding 0.97 kb PshAI–MfeI fragment in pHGPRP to create pHGHuPrP-129V or PrP-129M. In the resulting pHGHuPrP-129M clones, the signal-peptide sequence was still from mouse, but the rest of the PrP ORF and the first 76 bp after the stop codon were from human *PRNP* (prion protein gene) genomic DNA. The inserted 0.97 kb PshAI–MfeI fragment in pHGHuPrP-129V or PrP-129M was then sequenced with the primers HRM-R, HRM-F, and HP306R (CATGTTGGTTTTTGGCTTACTC). One error free 129V clone was chosen for the creation of TgWV mice. One error-free HuPrP-129M clone was used to generate a human $PrP^{N181Q/N197Q}$ ORF via PCR mutagenesis; the human $PrP^{N181Q/N197Q}$ transgene construct was then generated by inserting the human $PrP^{N181Q/N197Q}$ ORF into the NruI site of the pHGD3 plasmid that was made by replacing the mouse PrP ORF in the half-genomic PrP clone (pHGPRP) with the restriction sites for ClaI and NruI (*24*).

Generation, screening, and characterization of transgenic humanized TgWV and TgNN6h mice

The 12.2 kb HuPrP-129V (for TgWV mice) or $HuPrP^{N181Q/N197Q}$-129M transgene construct (for TgNN6h mice) was microinjected into fertilized FVB/NJ eggs, and planted into the oviducts of pseudopregnant CD-1 mice at the transgenic mouse facility of CWRU (Cleveland, OH), and the transgenic founders were bred repeatedly with FVB/*PRNP*$^{0/0}$ mice to obtain the TgWV and

TgNN6h mice on the FVB/*PRNP*$^{0/0}$ background, respectively, as described previously (*15, 24, 25*). TgNN6h or TgWV mice that are homozygous for the transgene were obtained by self-breeding between TgNN6h mice or between TgWV mice, respectively. Founder pups were screened by tail DNA PCR. PrP expression in the brain and other tissues of the Tg mice were determined by Western blot analysis using the monoclonal anti-PrP antibody 3F4.

Western blot analysis

Brain and skin samples were resolved on 15% Tris-HCl Criterion pre-cast gels (Bio-Rad) for SDS polyacrylamide gel electrophoresis at 150 V for ~80 min. The proteins on the gels were transferred to Immobilon-P membrane (PVDF, Millipore) for 2 h at 70V. The membranes were incubated for 2 h at room temperature with either 3F4 (1:40,000) or anti-C (1:10,000) as the primary antibody for probing the PrP molecule. Following incubation with horseradish peroxidase-conjugated sheep anti-mouse IgG at 1:5,000 for monoclonal antibodies or donkey-anti rabbit IgG at 1:6,000, the PrP bands were visualized on Kodak film by the ECL Plus in accordance with the manufacturer's protocol.

Hematoxylin & Eosin (H&E) staining and immunohistochemistry (IHC)

Fixed brain or skin tissues received from patients or animals were processed as described previously (*15*). Sections from the different brain areas or skin tissues were processed for hematoxylin & eosin staining and PrP immunohistochemistry with the anti-PrP monoclonal antibody (mAb) 3F4.

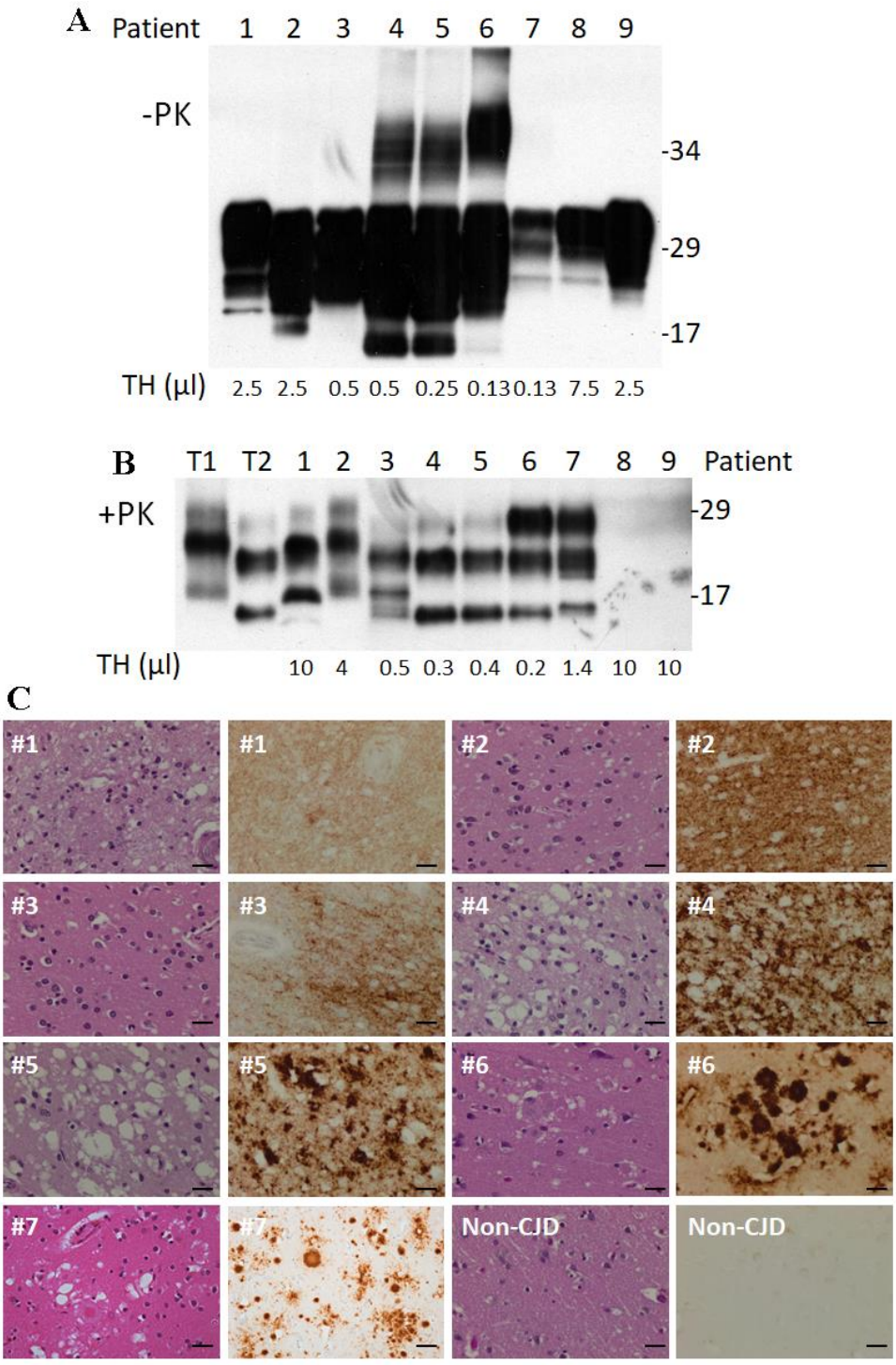

Fig. S1. Western blotting and neurohistology of autopsy brain samples from sCJD and vCJD patients. A and **B** show Western blot analysis of untreated (**A**) and PK-treated (**B**) brain PrP from CJD patients #1 through #7. Cases #8 and #9 are non-CJD controls. T1: sCJD PrP^{Sc} type 1 control; T2: sCJD PrP^{Sc} type 2 control. TH: the amounts of brain tissue loaded in each

well. The brain homogenates were treated with PK at 50 µg/ml for 1 h at 37°C. **C** shows hematoxylin & eosin (H&E) staining and immunohistochemistry with anti-PrP antibody 3F4 of the frontal cerebral cortex of brain sections from CJD patients 1 through 7. Non-CJD brain sections were used as controls. Patient: Pt. The bars represent 20 µm.

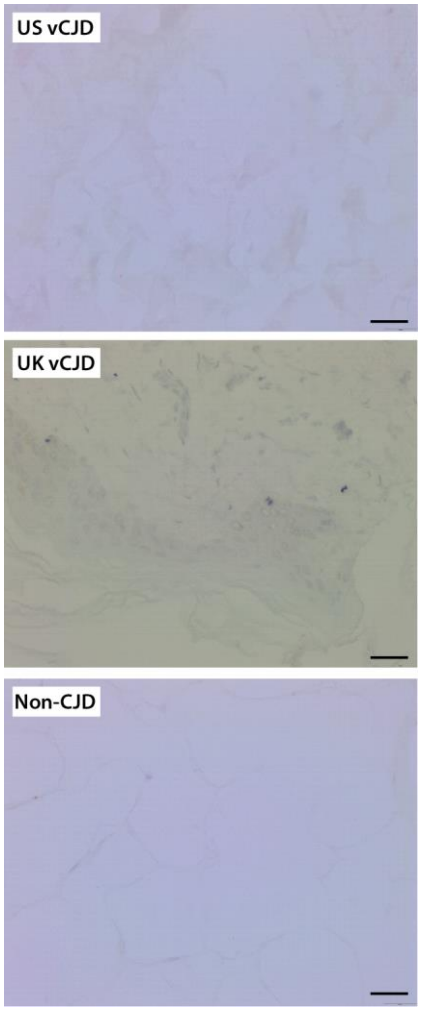

Fig. S2. Representative immunohistochemistry of skin samples from two vCJD patients and a non-CJD patient. Immunohistochemistry of skin samples from the two vCJD and a non-CJD patients probing with the anti-PrP antibody 3F4. The bars represent 50 µm.

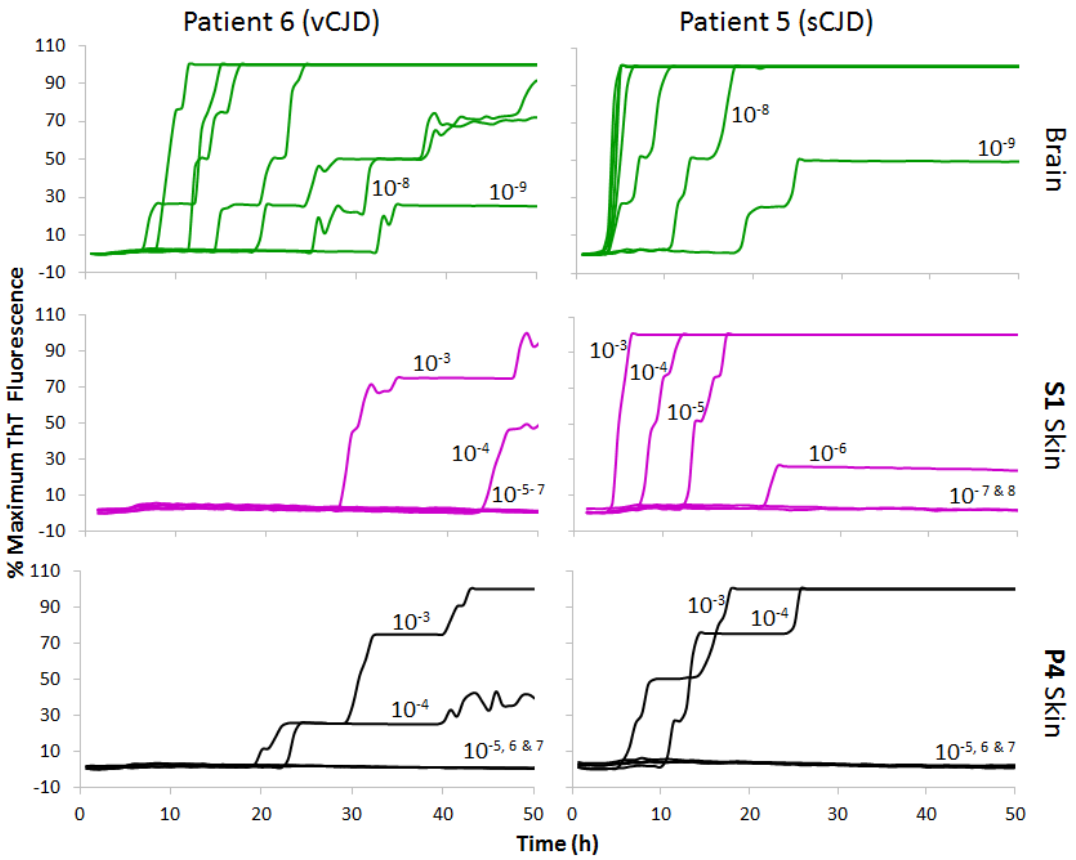

Fig. S3. Representative RT-QuIC end-point dilution analysis of brain and skin fractions from two CJD patients. Serial dilutions were used to seed quadruplicate RT-QuIC reactions with the BV rPrP^{sen} substrate. Reactions were seeded with dilutions of brain tissue (green), S1 skin fractions (magenta) or P4 skin fractions (black) from patient 6 (vCJD) and patient 5 (sCJD). For simplicity in the brain panels, only the traces from the 10^{-8} and 10^{-9} brain dilutions are marked even though traces from 10^{-3} to10^{-9} dilutions are shown. Similar results were seen in two independent experiments. Fluorescence readings from replicate wells were averaged (y-axis) and plotted as a function of time (x-axis).

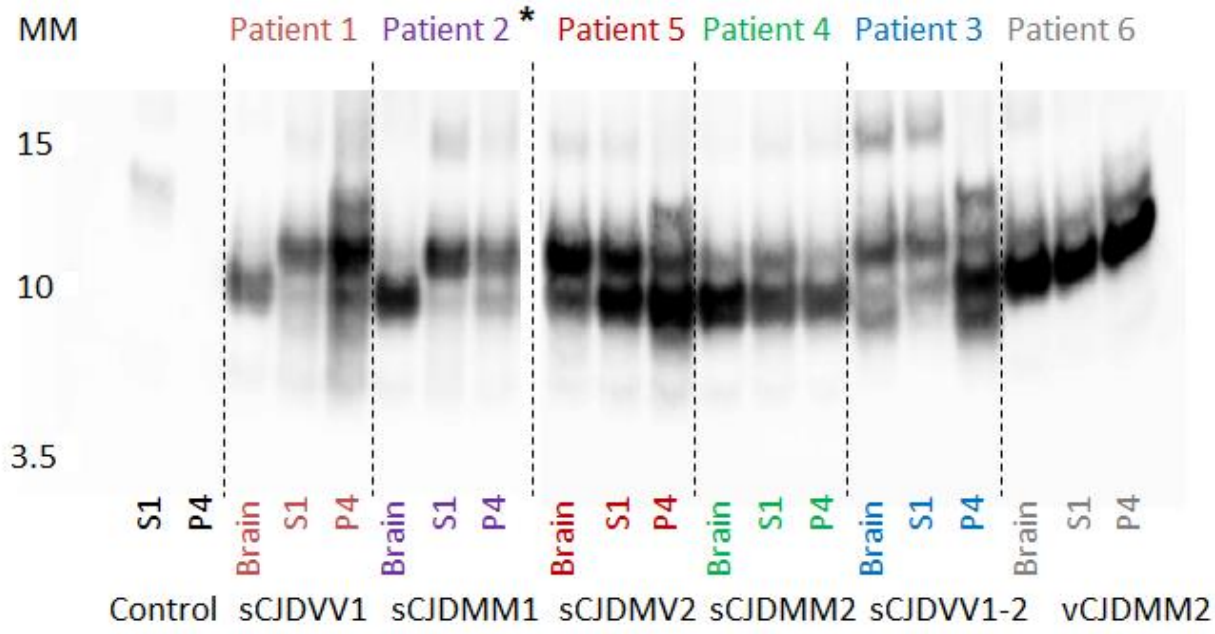

Fig. S4. Western blot analysis of bank vole rPrP^{res} RT-QuIC products from reactions seeded with brain or skin samples. Reaction products were digested with 10 µg/ml PK at 37°C for 1 h. BV rPrP^{res} conversion products were detected using C-terminal antiserum R20 (hamster PrP epitope residues 218-231). From left to right, BV rPrP^{Res} conversion products from reactions seeded with uninfected (Control; S1 and P4 skin samples from patient 9, Table S1), sCJD patients 1, 2, 5, 4 and 3 and vCJD patient 6. Western blotting analyses were performed at least twice for each brain and skin sample with similar results. Dotted lines delineate BV rPrP^{res} conversion products from reactions seeded with dilutions of brain, S1 and P4 fractions from the same patient. Positions of molecular mass markers (MM) are shown on the left in kDa. The asterisk marks the intersection between of two blots run under the same conditions that were combined to make this figure.

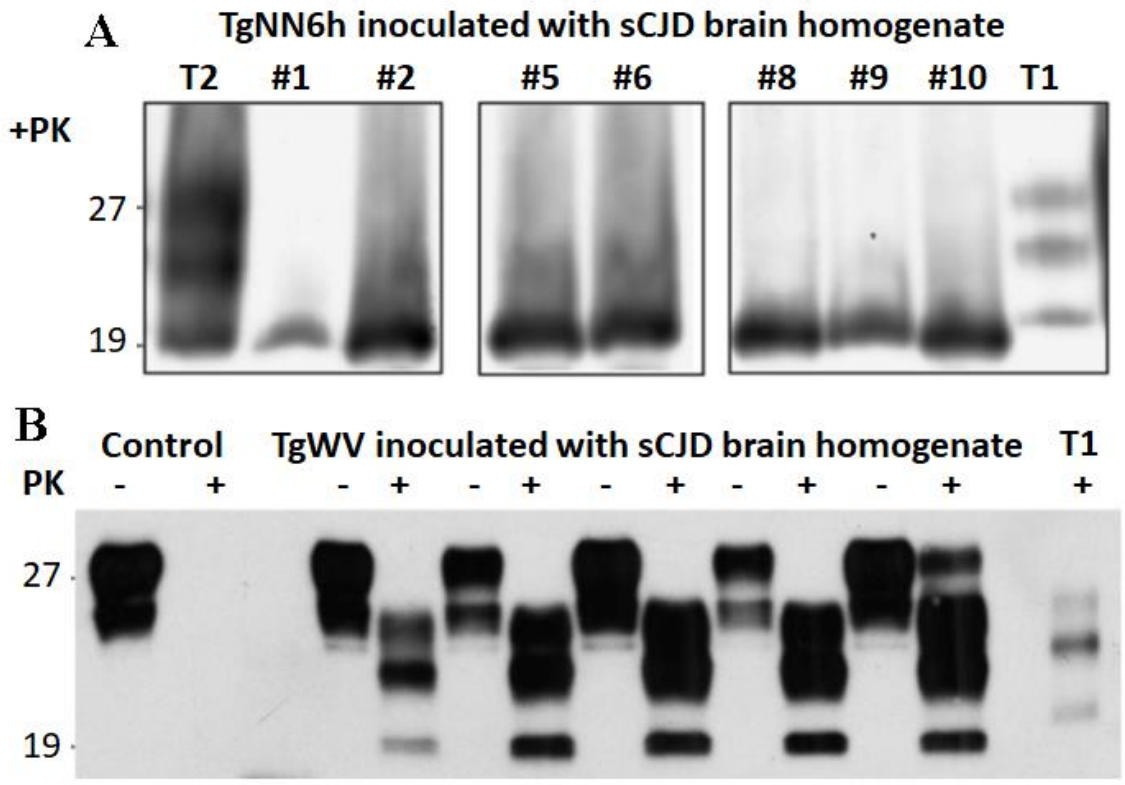

Fig. S5. Western blot analysis of brain samples from TgNN6h or TgWV mice inoculated intracerebrally with sCJDMM2 or sCJDVV2 brain homogenate, respectively. A: Western blotting and histology of brain tissues from TgNN6h mice inoculated i.c. with sCJDMM2 BH. **B:** Western blotting and histology of brain tissues from TgWV mice inoculated i.c. with sCJDVV2 BH. **a**, **c**, and **e**: Western blotting (**a**), H&E staining (**c**) and immunohistochemistry with the 3F4 antibody (**e**) of brain tissues from un-inoculated TgWV mice as controls; **b**, **d**, and **f**: Western blotting (**b**), H&E staining (**d**) and immunohistochemistry with the 3F4 antibody (**f**) of brain tissues from TgWV mice inoculated with sCJDVV2 BH. Western blots were probed with the 3F4 antibody.

Table S1. Summary of non-CJD cases.

Mean Age (years, SD)*	65.1 (14.2)
Male/Female (n, %)	9/6 (56.3/43.7)
Race (n, %)	
White	8(53.3)
Black	2 (13.3)
Hispanic	0 (0)
Mixed	0 (0)
Unknown	5 (33.3)
Final Diagnosis (n, %)	
Alzheimer's and agyrophilic grain disease	1 (6.7)
Hippocampal sclerosis	1 (6.7)
Progressive supranuclear palsy and Alzheimer's	1 (6.7)
Autoimmune encephalitis	1 (6.7)
Cardiac arrest	1 (6.7)
Infiltrating glioma	1 (6.7)
Macromastia	1 (6.7)
Metabolic astrocytosis	1 (6.7)
Pannus excision	2 (13.3)
Pulmonary hypertension	1 (6.7)
Stroke	1 (6.7)
Toxic shock syndrome	1 (6.7)
Unknown	2 (13.3)

*The skin samples were collected at autopsy or biopsy.