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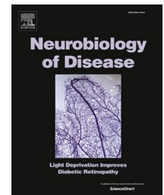

Formalin RT-QuIC assay detects prion-seeding activity in formalin-fixed brain samples from sporadic Creutzfeldt–Jakob disease patients

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A R T I C L E I N F O

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A B S T R A C T

Background: The neuropathology of sporadic Creutzfeldt–Jakob disease (sCJD) is usually investigated using formalin-fixed and formic acid-treated brain tissue. However, formalin and formic acid treatment can interfere with immunostaining of abnormal prion protein. Therefore, there is a need for biochemical methods other than immunostaining to investigate abnormal prion protein in postmortem tissue. We developed RT-QuIC to quantitate the seeding activity (SD_{50}) of sCJD brain tissue treated with formalin and formic acid.
Methods: We used endpoint RT-QuIC assays to analyze SD_{50} in formalin-fixed brain tissue from 19 sCJD patients (14 MM1 cases, 3 MM2-thalamic form [MM2T] cases and 2 MM2-cortical form [MM2C] cases) diagnosed according to Parchi's classification. We assessed SD_{50} in brains after incubation in formalin solution for over 1 month, and after treating formalin-fixed brain tissue with formic acid. We also examined how the SD_{50} values from formalin-fixed brain samples compared with neuropathological and immunohistochemical findings.
Results: The SD_{50} values of formalin-fixed brain samples from 14 MM1 cases, 2 MM2C cases, and 2 MM2T cases were $10^{7.77\pm0.57}$/g tissue, $10^{7.44\pm0.24}$/g tissue and $10^{6.00\pm0.77}$/g tissue, respectively. The average SD_{50} value in MM1 unfixed brains decreased by $10^{2.04}$ after formalin fixation for 1 month. In MM1 cases, after combined formalin and formic acid treatment, the SD_{50} value was reduced by approximately $10^{5.16}$ compared with that of unfixed tissue. The SD_{50} values of formalin-fixed tissue showed a consistent pattern with the neuropathological findings in most brain regions examined.
Conclusion: RT-QuIC enables the study of formalin-fixed brain tissue from sCJD patients that has not previously been amenable to analysis.

1. Introduction

Human prion diseases, or transmissible spongiform encephalopathies (TSEs), are fatal transmissible neurodegenerative diseases in humans (Prusiner et al., 1998) and are classified into sporadic, genetic and acquired forms; the most common being sporadic Creutzfeldt–Jakob disease (sCJD). The annual incidence rate of sCJD is 1.4 per million in Japan (Nozaki et al., 2010). Genetic prion disease is defined as a prion disease with causative mutations in the human Prion protein gene (*PRNP*) or a relevant family history, including Gerstmann–Straussler–-Scheinker disease, fatal familial insomnia (FFI), or genetic CJD (gCJD). The acquired forms are dura-associated CJD (dCJD) and variant CJD derived from bovine spongiform encephalopathy (Nozaki et al., 2010).

On the basis of the genotype at codon 129 of both *PRNP* alleles, the size of protease-resistant PrP^{Sc} fragments, and disease phenotype, sCJD is divided by Parchi's classification into six subtypes: MM1/MV1, VV2, MV2, MM2-cortical form (MM2C), VV1, and MM2-thalamic form (MM2T) (Parchi et al., 1999). Japan Surveillance Meetings reported that

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MM1 and MV1 patients constitute about 90% of the Japanese sCJD population, and include cases previously classified as typical CJD of the myoclonic type or as Heidenhain variant. Thus, the genotype distribution at codon 129 of PrP in sCJD disease in Japan reveals a higher proportion of methionine homozygotes (96.8%) (Saborio et al., 2001). The general Japanese population also presents with a higher frequency of codon 129 homozygosity (methionine homozygotes: 0.92, heterozygotes: 0.08, valine homozygotes: 0) than populations in European countries (methionine homozygotes: 0.37–0.49, heterozygotes: 0.42–0.49, valine homozygotes: 0.08–0.15).

The infectious agent, or prion, of TSEs appears to be composed of an abnormal prion protein (PrP^{Sc}). PrP^{Sc} is formed post-translationally from the normal cellular prion protein (PrP^{c}), which in purified form can resemble amyloid fibrils. PrP^{Sc} induces the polymerization and conformational conversion of PrP^{c} to infectious PrP^{Sc} or to PrP^{Sc}-like partially protease-resistant PrP forms in a variety of in vitro reactions (Prusiner et al., 1998).

In vitro PrP^{Sc} amplification techniques, including protein misfolding cyclic amplification (PMCA) (Atarashi et al., 2008), amyloid seeding assay (ASA), and quaking-induced conversion (QuIC) (Atarashi et al., 2011) enable the highly sensitive detection of PrP^{Sc}. QuIC assays use recombinant normal prion protein (*rPrP-sen*) as a substrate to amplify very slight amounts of PrP^{Sc} with intermittent automated shaking, which can be performed more easily than sonication used in PMCA. We recently developed a new in vitro amplification technology, designated “real-time quaking-induced conversion (RT-QuIC)”, for detecting prion-seeding activity from many organs, including central nervous system (CNS) tissues and non-CNS tissues from sCJD patients (Takatsuki et al., 2015). Previously, we developed the RT-QuIC assay for detection of very small amounts of abnormal PrP in tissue and body fluids. These studies demonstrated that RT-QuIC is more sensitive than bioassays. Importantly, endpoint RT-QuIC can be used to evaluate human prion-seeding activity in brains from patients with human prion disease (Akagi et al., 2018).

The neuropathology of sCJD is usually investigated using formalin-fixed and formic acid-treated brain tissue. The neuropathology of sCJD is characterized by the presence of spongiform changes, neuronal loss and the accumulation of PrP^{Sc} deposition. Pathologists treat tissue with formic acid to reduce prion infectivity. Furthermore, to enhance the immunoreactivity for PrP^{Sc}, many researchers use a combination of formic acid and autoclaving pretreatment. In some cases, even if western blotting detects similar levels of abnormal prion protein in the unfixed brain tissue, the pretreatment methods for immunostaining can alter the staining property of the tissues differently. This limitation has typically resulted in qualitative, rather than quantitative, pathological evaluations. Once brain tissue is fixed with formalin, its usefulness is limited for uses other than neuropathological analysis. It is highly desirable to be able to perform quantitative and biochemical analyses on formalin-fixed brains. Therefore, in the present study, we used our previously developed RT-QuIC method to quantitate the prion-seeding activity (SD_{50}) in formalin-fixed brain tissue from sCJD patients.

2. Materials and methods

2.1. Human samples (Table 1)

We used endpoint RT-QuIC assays to analyze 19 brain samples obtained from definite cases of 19 sCJD patients: 14 MM1 cases (10 cases without formalin treatment and 4 cases fixed with formalin), 3 MM2T cases, and 2 MM2C cases. Sporadic CJD was diagnosed according to Parchi’s classification based on the genotype at codon 129 of *PRNP* (methionine homozygous [MM], valine homozygous [VV], or heterozygous [MV]), and the physicochemical properties of PrP^{Sc}. For formalin-treated tissue, the entire cortex was treated with formalin for 1 month, and the samples that received combined treatment were subsequently exposed to 99% formic acid for 1 h.

Table 1
Summary of sporadic Creutzfeldt–Jakob disease patients.

Patient number	Sex	Age onset	sCJD subtype based on Parchi’s classification	Log SD50/g tissue in native brain (mean ± SD)	Log SD50/g tissue in formalin-fixed brains (mean ± SD)	Log SD50/g tissue in formalin-fixed brains after formic acid treatment (mean ± SD)
1	Female	69	MM1	10.38 ± 0.18	7.88 ± 0.53	4.75 ± 0.35
2	Female	80	MM1	10.25 ± 0.35	7.88 ± 0.17	5.13 ± 0.18
3	Female	50	MM1	10.00 ± 0.00	7.50 ± 0.35	4.75 ± 0.35
4	Male	68	MM1	10.00 ± 0.35	7.75 ± 0.35	5.13 ± 0.18
5	Female	81	MM1	10.13 ± 0.18	7.63 ± 0.53	5.00 ± 0.35
6	Male	86	MM1	9.75 ± 0.00	7.63 ± 0.18	4.88 ± 0.18
7	Male	64	MM1	9.88 ± 0.18	7.38 ± 0.17	4.63 ± 0.53
8	Female	84	MM1	10.00 ± 0.35	7.75 ± 0.35	5.13 ± 0.18
9	Male	79	MM1	10.13 ± 0.18	7.88 ± 0.53	4.50 ± 0.35
10	Female	72	MM1	9.50 ± 0.00	7.13 ± 0.17	4.50 ± 0.35
11	Female	74	MM1	N.A.	7.75 ± 0.71	5.00 ± 0.00
12	Male	73	MM1	N.A.	8.38 ± 0.53	4.75 ± 0.35
13	Male	75	MM1	N.A.	8.13 ± 0.89	5.00 ± 0.00
14	Male	64	MM1	N.A.	8.13 ± 0.18	5.25 ± 0.00
15	Female	68	MM2T	N.A.	5.25 ± 0.00	3.50 ± 0.00
16	Male	40	MM2T	N.A.	5.88 ± 0.18	3.88 ± 0.18
17	Female	69	MM2T	N.A.	6.88 ± 0.53	4.50 ± 0.35
18	Male	82	MM2C	N.A.	7.50 ± 0.35	4.88 ± 0.17
19	Male	67	MM2C	N.A.	7.38 ± 0.17	4.62 ± 0.18

Clinical data and the SD50 concentrations in formalin-fixed brain homogenates from patients with prion disease. None of the patients have a *PRNP* mutation.
Based on the genotype at codon 129 on both *PRNP* alleles, the size of protease-resistant PrP^{Sc} fragments, and disease phenotype, we divided sporadic CJD into six subtypes:
sCJDMM1/sCJDMV1, sCJDVV2, sCJDMV2, sCJDMM2-cortical form, sCJDVV1, and MM2-thalamic form according to Parchi’s classification.
The MM1 patients: The pion seeding values of sCJD’s brain decreased 2.36 log after treating with formalin (SD50: $10^{10\pm0.29} \rightarrow 10^{7.64\pm0.36}$) which got further reduced decreased 2.8 log (SD50: $10^{7.64\pm0.36} \rightarrow 10^{4.84\pm0.34}$) after treating with formalin and formic acid of 10 cases MM1 (patients 1–10). the average of SD50 of 14 cases MM1 (patients 1–14) decreased 2.89 log (SD50: $10^{7.77\pm0.46} \rightarrow 10^{4.88\pm0.32}$) after combining formalin and formic acid treatment.
The MM2 patients: The SD50 values of sCJD’s brain of 2 cases MM2C (patients 18–19) decreased 2.69 log (SD50: $10^{7.44\pm0.24} \rightarrow 10^{4.75\pm0.2}$), three cases MM2T (patients 15–17) reduced 2.04 log (SD50: $10^{6.00\pm0.77} \rightarrow 10^{3.96\pm0.49}$) after formalin and formic acid treatment.
Data are presented as means ± standard deviation. MM2C: cortical form of MM2.
MM2T: thalamic form of MM2.
N.A.: not available.

To examine prion distribution in different parts of the brain, we analyzed prion-seeding activity by using endpoint RT-QuIC assay in six different regions (frontal, temporal, parietal, occipital, thalamus, and cerebellum) of MM1 ($n = 2$), MM2T (n = 2), and MM2C ($n = 1$) samples. We included 105 non-CJD brain specimens as negative controls.

2.2. Preparation of tissue homogenate (non-fixed brains)

Brain tissue was subjected to RT-QuIC assay to evaluate SD_{50} (Akagi et al., 2018). To prevent contamination from brain tissue into other samples, we used single-use disposable tubes and beads, and all procedures were performed on different days. Brain tissue samples were homogenized in 10% (*w/v*) ice-cold phosphate-buffered saline supplemented with a protease inhibitor mixture (Roche, Mannheim, Germany) using a multi-bead shocker (Yasui Kikai, Osaka, Japan). The samples were clarified by centrifugation at 6000 rpm for 2 min and stored at −80 °C.

2.3. Preparation of tissue homogenate (formalin-fixed and formic acid-treated brains)

After treatment in formalin solution for over 1 month, each brain sample was cut into small pieces, each weighing approximately 50 to 200 mg. The formalin-fixed brain samples were subjected to RT-QuIC assay to evaluate SD_{50}. To prevent contamination from brain tissue into other samples, we used single-use disposable tubes and beads, and all procedures were performed on different days. Brain tissue samples were homogenized in 10% (*w/v*) ice-cold phosphate-buffered saline supplemented with a protease inhibitor mixture (Roche, Mannheim, Germany) using a multi-bead shocker (Yasui Kikai, Osaka, Japan). The samples were clarified by centrifugation at 10,000 $\times g$ for 2 min and stored at −80 °C.

Alternatively, some formalin-fixed brain samples were subsequently immersed in 20 ml 99% formic acid for 1 h and then washed three times with 100 ml sterile distilled water. These samples were then processed using the same procedures as for formalin-fixed brain samples.

2.4. Endpoint RT-QuIC (Fig. 1)

Analysis of the formalin RT-QuIC assays and endpoint formalin RT-QuIC assay was similar to the previously described unfixed brain RT-QuIC assays (Takatsuki et al., 2015). In brief, the RT-QuIC reaction mix was composed of 50 mM PIPES (pH 7.0), 500 mM NaCl, 10 μμM Thioflavin T (ThT), 0.1 mM ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA), and recombinant human PrP (residues 23–231 with 129 M). Aliquots of the reaction mix (90 μl) were loaded into each well of a black 96-well plate with a clear bottom (Nunc 96 well; Sigma-Aldrich, USA) and mixed with 10 μl of brain homogenate. Four to eight replicates of each diluted sample were measured, and PrP amyloid formation was monitored for 48 h. We calculated the 50% seeding dose (SD_{50}) in unfixed brains, formalin-fixed brains and formic acid-treated brains using the Spearman–Kärber method.

We define a positive reaction by RT-QuIC assay when the following three conditions are met: (1) the absorbance rises within 150 cycles, and after reaching the maximum absorbance, the level of the absorbance is stable; (2) the maximum absorbance is more than six times higher than the starting absorbance; and (3) only recombinant protein was used to maintain the quality of the lot when the prepared recombinant protein showed an $SD_{50} \geq 10^7$ by RT-QuIC assay in the brain of MM1-type sCJD.

The assay was repeated at least twice, and Spearman–Kärber analysis was used to estimate a seeding dose (SD_{50}). The SD_{50} was calculated using the following formula: $xp = 1 + 1/2d - dgp$, where $xp = 1$ represents the highest log dilution giving all positive responses, d is the log dilution factor, p is the proportion positive at a given dose, and gp is the sum of values of p for $xp = 1$ and all higher dilutions (Wilham et al., 2010).

2.5. Neuropathological examination

A postmortem study was performed within 22.5 h after death. The brain and upper cervical spinal cord were fixed in 20% neutral-buffered formalin for 4 weeks, and tissue blocks were immersed in 95% formic acid for 1 h to inactivate prion infectivity. The specimens were then embedded in paraffin and cut in 9-μm-thick sections. The sections were deparaffinized in Lemosol, rehydrated through an ethanol gradient, and stained. For routine neuropathological examinations, sections were subjected to hematoxylin and eosin (HE) and Klüver-Barrera (*KB*) staining and modified Gallyas-Braak silver staining. We evaluated the severity of the neuropathological changes as mild, moderate or severe, according to Parchi's pathological staging for sCJD (Parchi et al., 1999).

Immunohistochemical analysis was performed with a mouse monoclonal antibody against PrP (3F4, dilution 1:100; Dako, Glostrup, Denmark) after hydrolytic autoclaving for antigen retrieval. PrP immunostaining was conducted as previously described (Tateishi et al., 1996). Peroxidase-conjugated streptavidin was visualized with 3, 3′-diaminobenzidine (DAB; Wako Pure Chemical Industries, Japan) as the final chromogen. Immunostained sections were lightly counterstained with HE staining.

2.6. Ethics approval and consent to participate

The protocol for investigation was approved by the Ethics Committee of Nagasaki University Hospital (ID: 10042823), and the study was registered with the University Hospital Medical Information Network (ID: UMIN000003301) and (ID: UMIN000038398). The protocol was also granted ethical approval for the use of brain tissue by the Japan Surveillance Unit for human prion diseases.

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS® Statistics Version 22 (IBM Japan, Tokyo). We used the Mann–Whitney *U* test for comparisons of SD_{50} between sCJD patients.

3. Results

3.1. Analysis of formalin-fixed brain tissue in the frontal lobe from all sCJD patients by endpoint RT-QuIC assay (Table 1 and Fig. 1, Supplementary Fig. 1 [SF 1], and Supplementary Fig. 2 [SF 2])

We analyzed brain samples from all patients (unfixed, formalin-fixed and formic acid-treated tissue) by endpoint RT-QuIC assay. We first analyzed patient 1 (sCJD MM1) alone to gain a better understanding of the calculation method, which we showed in detail as a representative. Brain homogenate was diluted from 1×10^{-5} to 1×10^{-10} and subjected to endpoint RT-QuIC to quantitate seeding activity. The positive reaction percentage decreased in a sigmoidal curve within the dilution range and the SD_{50} was calculated using the Spearman–Kärber method (Fig. 1 and SF 1).

We determined the optimal time for formalin fixation before proceeding with the experiment. Generally, formalin fixation of brain tissue is performed for 3–4 weeks. We measured the effect of time in formalin on SD_{50} of the untreated brain. SD_{50} at 1 week, 2 weeks, 3 weeks, 4 weeks and 3 months of formalin fixation were 10.13, 10.08, 8.24, 7.88 and 7.85, respectively (SF2).

As shown in Fig. 1, unfixed brain homogenate from patient 1 had a concentration of $10^{10.38\pm0.17}$ SD_{50} per gram of tissue, the formalin-fixed tissue contained $10^{7.88\pm0.53}$ SD_{50} per gram of tissue equivalent, and the formalin-fixed and formic acid-treated tissue had $10^{4.75\pm0.35}$ SD_{50} per gram of tissue equivalent. Our analysis showed that we successfully determined prion-seeding activity in formalin-fixed and formic acid-treated sCJD brain tissue. It also showed that formalin treatment of

the MM1 sCJD tissue for 1 month decreased the SD_{50} value by 2.5 log, and formalin treatment with subsequent exposure to >99% formic acid for 1 h decreased it by 5.63 ($10^{10.38}$ in unfixed tissue vs. $10^{7.88}$ after formalin fixation vs. $10^{4.75}$ after formic acid treatment) compared with the unfixed tissue log SD_{50}.

To confirm the above results, we determined the SD_{50} values for unfixed brain homogenate, formalin-fixed brain tissue and formalin-fixed and formic acid-treated brain tissues from 10 MM1 cases (patients 1–10). The prion seeding values of sCJD brains decreased by 2.36 log after formalin treatment (SD_{50}: $10^{10\pm0.29}$ vs. $10^{7.64\pm0.36}$), and further decreased by 2.8 log (SD_{50}: $10^{7.64\pm0.36}$ vs. $10^{4.84\pm0.34}$) after subsequent formic acid treatment (Table 1, SF 1 and SF 3).

Using the same method, we analyzed 19 sCJD patients (Table 1), comprising 14 patients with MM1, 3 patients with MM2T and 2 patients with MM2C. The averages of log10 SD_{50} in formalin-fixed brains of MM1, MM2T and MM2C patients were 7.77, 6.00 and 7.44, respectively. After the formic acid treatment, values for MM1, MM2T and MM2C patients were 4.88, 3.96 and 4.75, respectively. MM1 and MM2C patients exhibited a similar level of seeding activity ($10^{7.38–8.38}$/g), and the SD_{50} of MM2T patients was lower than those of MM1 and MM2C patients.

3.2. Analysis of prion distribution in six brain areas by endpoint RT-QuIC assay and the neurological findings of an MM2C patient (Fig. 2a)

To measure prion-seeding activity throughout the brain, we collected six representative sites and measured prion-seeding activity in those locations (frontal lobe, temporal lobe, parietal lobe, occipital lobe, thalamus and cerebellum). In an MM2C patient (patient 18), the spongiform change typically consisted of large and coarse vacuoles. PrP immunohistochemistry showed a coarse pattern of staining (Fig. 2a).

In the MM2C patient, the level of log SD_{50}/g tissue in the six areas ranged from 6.63 to 8.75; that of the temporal lobe was higher than that of the frontal lobe, occipital lobe, parietal lobe and thalamus. Fig. 2 shows that the level of log SD_{50}/g tissue was the highest in the temporal lobe ($10^{8.75\pm0.35}$/g tissue), lower in the occipital lobe ($10^{8.5\pm0.35}$/g tissue) and parietal lobe ($10^{8.00\pm0.00}$/g tissue) and lowest in the frontal lobe ($10^{7.5\pm0.35}$/g tissue), thalamus ($10^{7.5}$/g tissue) and cerebellum ($10^{6.63\pm0.53}$/g tissue).

In the neuropathological findings of patient 18, PrP^{Sc} accumulation was highest in the temporal lobe, lower in the frontal lobe, occipital lobe, and parietal lobe, and lowest in the thalamus and cerebellum. Therefore, the neurological findings were consistent with the SD_{50} values in the MM2C patient.

3.3. Analysis of HE staining, PrP immunostaining and prion distribution, endpoint RT-QuIC assay in an MM1 patient and an MM2T patient (Fig. 2b and Table 2)

We measured SD_{50} in the frontal lobe and thalamus of formalin-fixed tissue from an MM1 patient and MM2T patient. We also examined the neuropathological findings of HE staining and PrP immunostaining in the frontal lobe and thalamus.

In the HE stainings of the sCJD MM1 patient, the spongiform change was characterized by small and typical vacuoles in the neuropil of the frontal lobe. Synaptic-type PrP deposition in the frontal lobe and thalamus was observed by PrP immunostaining in the MM1 patient, and PrP deposition in the frontal lobe was higher than that in the thalamus. Additionally, the log SD_{50} in the frontal lobe (8.13) was higher than that in the thalamus (7.5). Therefore, we observed a positive relationship between SD_{50} and neuropathological findings in the MM1 patient (Fig. 2b).

There was prominent atrophy of the thalamus and inferior olive with only minor pathologic changes in the other areas of the MM2T brain. Table 2 shows that the prion-seeding activity in the MM2T patient was not only concentrated in the thalamus, with a range of 6.50–7.38 log SD_{50} per gram, but had also spread to the cerebral cortex and the cerebellum, with a range of 5.6–7.13 log SD_{50} per gram. As shown in Fig. 2b, very mild spongy changes in the frontal lobe and the thalamus were observed with HE staining. We also noticed very mild PrP deposition in the thalamus and the frontal lobe by PrP immunostaining.

3.4. Analysis of white and gray matter in the frontal lobe of sCJD formalin-fixed brains (Fig. 3)

We measured SD_{50} in formalin-fixed brain tissue from all sCJD patients, and we analyzed prion-seeding activity of the white matter and gray matter from the frontal lobe of all sCJD patients. In all patients, the prion seeding values were higher in gray matter than in white matter. (Fig. 2 and Supplementary Table 1).

After formalin fixation, the average log SD_{50} of 14 MM1 patients was 7.93 ± 0.38 and 7.88 ± 0.35 in gray matter and white matter, respectively. In 3 MM2C patients, the log SD_{50} was 7.63 ± 0.32 and 7.19 ± 0.51 in gray matter and white matter, respectively. And in 3 MM2T cases, the log SD_{50} was 6.67 ± 0.35 and 6.42 ± 0.47 in gray matter and white matter, respectively. Therefore, the difference between gray matter and white matter was the smallest in the MM1 cases, at approximately 0.05, larger in the MM2T cases, at approximately 0.25, and the largest in the MM2C case, at 0.44. After further treatment with formic acid, the difference between gray matter and white matter was the smallest still in the MM1 cases (approximately 0.1), and in MM2T and MM2C cases was 0.28 and 0.25, respectively.

4. Discussion

To conduct neuropathological analyses in patients with human prion disease, the brain tissue is routinely fixed with formalin and treated with formic acid to reduce the infectivity of abnormal prion proteins. Performing biochemical analyses on formalin-fixed brain tissue is difficult because proteins are rapidly denatured when the brain is immersed in formalin solution. In this study, we successfully measured prion-seeding activity in formalin-fixed brain samples and in formic acid-treated formalin-fixed brain using RT-QuIC. To the best of our knowledge, no previous studies have reported comparisons of infectivity when tissue is fixed with formalin and treated with formic acid, other than the infection experiment (Tateishi et al., 1996); the present study is the first to report how infectivity changes in treated brain tissue of sCJD patients.

The level of SD_{50} decreased with the time that brains were immersed in formalin solution (ranging from $10^{10.34}$ to $10^{7.88}$; Table 1, Fig. 1 and SF 2b). The SD_{50} level also decreased with formic acid treatment after formalin fixation (from $10^{7.88}$ to $10^{4.75}$). Formalin treatment of MM1 brain tissue for 1 month decreased the SD_{50} value by 2.36 log compared with unfixed tissue (SF3). Furthermore, the SD_{50} of unfixed tissue decreased considerably 1 week from the start of formalin fixation (SF 3b). The SD_{50} was stable after 3 weeks from the start of formalin fixation (SF 2). For neuropathological assessments, brain tissue is routinely fixed in formalin for at least 3 weeks, and our findings indicate that this duration is adequate to reach stable SD_{50} values.

Subsequent >99% formic acid treatment after formalin fixation decreased the value of log10 SD_{50} in MM1, MM2C and MM2T cases by approximately 2.8, 2.69 and 2.04, respectively, compared with unfixed tissue. Therefore, the prion-seeding activity of MM1 cases with subsequent exposure to formic acid after formalin fixation was decreased by 5.16 logs compared with the MM1 unfixed brain tissue.

Bioassays for human prion disease can be performed in humanized knock-in mice and sometimes can be used in 263 K mice or VM mice. In a previous study, the infectivity titer "infectious dose $(ID)_{50}$" of unfixed brain from VM mice terminally affected with the 301 V strain of mouse-passaged BSE agent was approximately $10^{9–10}$ ID_{50}/g; after formic acid treatment of paraformaldehydelysine-periodate (PLP)-fixed brain, infectivity was $<10^{2.5}$ ID_{50}/g, and after formic acid treatment of formalin-fixed brain, infectivity was $<10^{3.0}$ ID_{50}/g (Taylor et al., 1997).

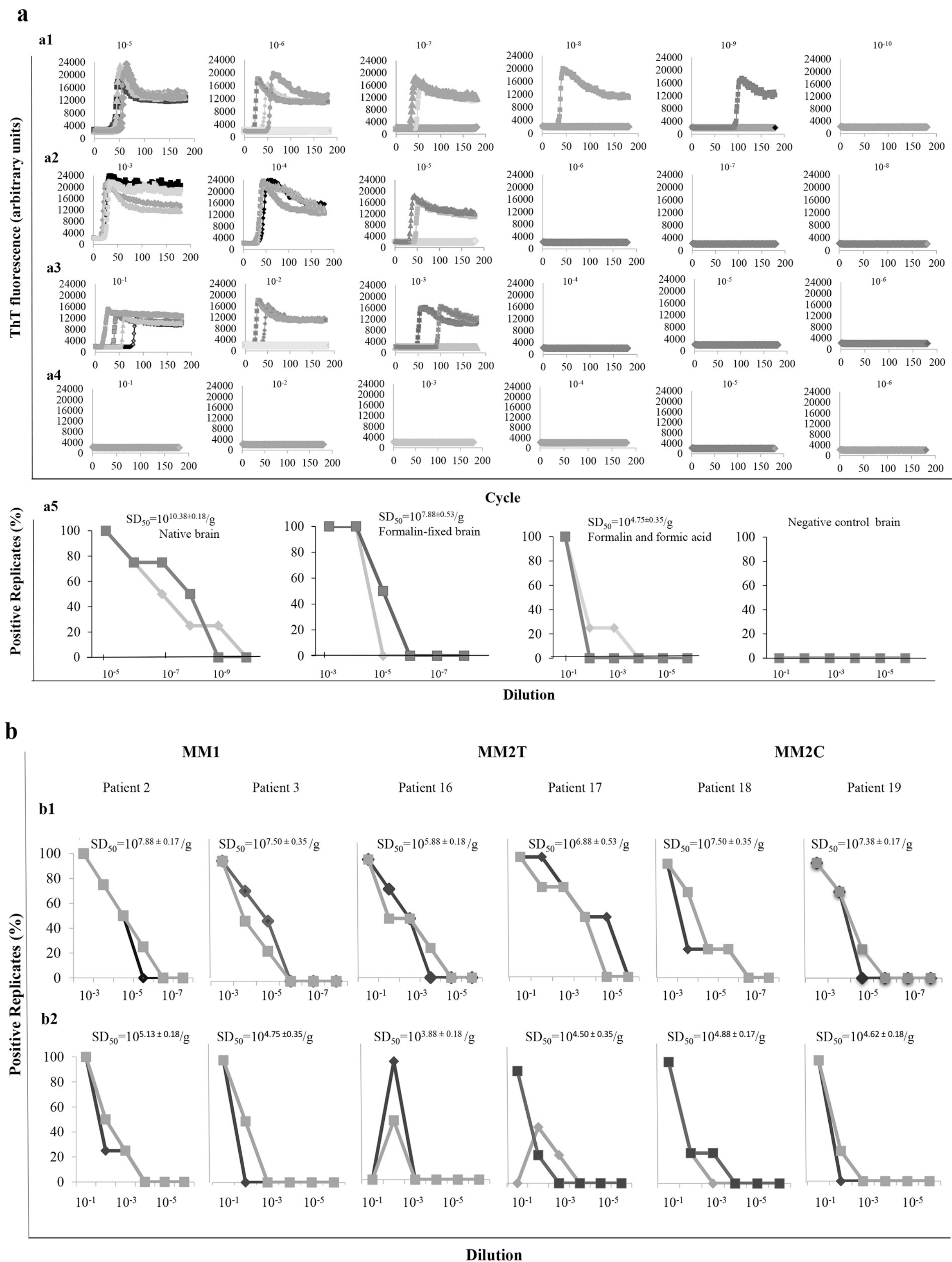

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Fig. 1. Quantitation of SD_{50}in patient brains by endpoint RT-QuIC.
(a). Quantitation of SD_{50} in the patient 1 brain.
(a1) Native brain homogenates were diluted (1×10^{-5} to 1×10^{-10}) and subjected to the RT-QuIC reaction. ThT fluorescence was elevated at dilutions from 1×10^{-5} to 1×10^{-10}.
(a2) Formalin-fixed brain homogenates were diluted (1×10^{-3} to 1×10^{-8}) and subjected to the RT-QuIC reaction. ThT fluorescence was elevated at dilutions from 1×10^{-3} to 1×10^{-8}.
(a3) Formalin and formic acid-treated brain homogenates were diluted (1×10^{-1} to 1×10^{-6}) and subjected to the RT-QuIC reaction. ThT fluorescence was elevated at dilutions from 1×10^{-1} to 1×10^{-6}.
(a4) Negative control reactions were seeded in quadruplicate with a 1×10^{-6} dilution of brain tissue from a negative control brain.
(a5) Reduction of seeding activity by treatment with formalin and formic acid in patient 1 using endpoint RT-QuIC. Prion seeding values of an sCJD brain were decreased by 2.5 log after formalin fixation and by approximately 5.63 log SD_{50} following combined formalin fixation and formic acid treatment.
(b). Quantitation of SD_{50} in six patient brains (2MM1, 2MM2T, and 2MM2C).
(b1) SD_{50} of a formalin-fixed brain determined by endpoint RT-QuIC.
(b2) Endpoint RT-QuIC to quantitate the SD_{50} in a formalin-fixed and formic acid-treated sample.
RT-QuIC assays were performed twice.

Although ID_{50} and SD_{50} correlate, the values do not represent the same information. In addition, data obtained by qualitative assay after a similar treatment of 301 V supports our results (Taylor, 1995). A previous study reported that the infectivity titer in formalin- or PLP-fixed mouse brains infected with the 301 V BSE agent were reduced by around 6 logs following treatment with formic acid compared with unfixed brains. Similarly, formic acid-treated mouse brain infected with the ME7 strain of scrapie agent showed a reduction of around 5 logs compared with unfixed brain (Taylor et al., 1997). Another study in formalin-fixed rodent brain infected with CJD agents showed that the infectivity titers of scrapie were reduced by approximately 9 logs (from $10^{10.2}$ to $10^{1.3}$), and by approximately 6 logs (from $10^{8.5}$ to $10^{2.3}$) after an exposure to >96% formic acid for 1 h (Brown et al., 1990). In the present study, the log10 SD_{50}/g tissue values of brain samples from MM1, MM2C and MM2T patients were 7.77, 7.44 and 6.00, respectively, after formalin fixation for 1 month, and were approximately 4.88, 4.75 and 3.96, respectively, after combined treatment with formalin and formic acid. The changes reported in the infectivity titer (ID_{50}) of PLP-fixed mouse brain after formic acid treatment were consistent with the changes in prion-seeding activity in human brain in the present study. Infectivity decreased after formalin and formic acid treatment in previous studies in mouse brains infected with the 301 V BSE agent or ME7 strain of scrapie agent by 5–6 logs. These findings show that the reduction in infectivity after combined formalin and formic acid treatment is similar to the reductions in prion-seeding activity found in the present study using endpoint RT-QuIC assays.

We assumed that an SD_{50} titer of 7–7.5 is the limiting point of transmission for humanized knock-in mice (Takatsuki et al., 2016). Therefore, we conclude that in humanized knock-in mice, the formalin-fixed brain would be infectious. In the tissue in the present study, which were treated with a combination of formalin and formic acid, the prion-seeding activity in the treatment of formalin and formic acid was 100,000 times lower than that of the unfixed brain. Therefore, formic acid treatment is a useful method to reduce the risk of exposure of laboratory workers to highly infectious prions.

In the present study, we compared PrP deposition and prion-seeding activity (SD_{50}). The level of SD_{50} in formalin-fixed tissue was consistent with the amount of PrP^{Sc} accumulation, detected by PrP immunostaining (Fig. 2). PrP immunostaining of MM1 brains showed that the amount of PrP^{Sc} accumulation was similar in the cerebral cortex and cerebellum, and was lower in the brainstem (substantia nigra, midbrain periventricular gray, locus coeruleus and medulla) and hippocampus. Additionally, immunostaining showed slightly more PrP^{Sc} accumulation in the occipital lobe (SD_{50}: 8.75) than in the frontal lobe, temporal lobe and parietal lobe (SD_{50}: 7.63–7.88) (Table 2).

The amount of the PrP^{Sc} accumulation detected by PrP immunostaining in the MM2C brains was similar in the cerebral cortex and cerebellum, and lower in the brainstem. Immunostaining also showed that PrP^{Sc} accumulation was at the same level in all lobes of the cerebral cortex in this MM2C patient. However, the SD_{50} assay in the MM2C patient showed that SD_{50} in the frontal lobe (SD_{50}: 7.5) was lower than that in the occipital lobe, temporal lobe and parietal lobe (SD_{50}: 8–8.75). These findings indicate that there was a consistent relationship between SD_{50} and neuropathological findings throughout the cerebral cortex, except for in the frontal lobe.

In a previous study, the levels of PrP^{Sc} accumulation detected by PrP immunostaining in MM2T brains were similar throughout the cerebral cortex and lower than those in MM1 brains (Tateishi et al., 1996). Additionally, there was more PrP^{Sc} accumulation in the thalamus and cerebellum of MM2T brains than in the cerebral cortex, including the frontal lobe, temporal lobe and parietal lobe. Our SD_{50} assay results in the MM2T (patient 16) showed that the SD_{50} of the thalamus (SD_{50}: 6.5) and cerebellum (SD_{50}: 6.63) were higher than those of the frontal lobe, temporal lobe and parietal lobe (SD_{50}: 5.6–5.88). Also, PrP^{Sc} accumulation was higher in the thalamus than in other areas of this MM2T brain (Fig. 2). Thus, there was a consistent pattern between SD_{50} and the neuropathological findings throughout the cerebral cortex, except for in the occipital lobe. These results suggest that the SD_{50} for formalin-fixed brain tissue is compatible with the neuropathological findings in multiple sCJD subtypes.

The SD_{50} values in formalin-fixed sCJD MM1 brain were fairly consistent between patients, ranging from 7 to 7.5. However, PrP^{Sc} immunostaining was weaker in some MM1 patients than in others. This variation is likely due to differences in storage conditions of formalin-fixed brains, or differences in the degree of infiltration of formalin from brain to brain. Because of these limitations of PrP immunostaining, SD_{50} calculation by RT-QuIC assay is a useful alternative to quantify abnormal prion proteins. It is important to note that PrP immunostaining allows for the quantitative analysis of an individual sCJD patient, whereas other neuropathological techniques to assess SD_{50} can only provide semi-quantitative data of individual patients.

We evaluated the brains of three subtypes of sCJD patients, and the neuropathological findings indicated that the MM2T cases were the pure type of MM2T (Fig. 2b). In the pure type of MM2T, the amount of PrP^{Sc} in the cerebral cortex is lower than that in the thalamus according to the neuropathological and SD_{50} analyses (Parchi et al., 1999). In a previous study, the levels of PrP^{Sc} accumulation detected by PrP immunostaining in MM2T brains were similar throughout the cerebral cortex and lower than those in MM1 brains (Tateishi et al., 1996). In the present study, the level of SD_{50} in formalin-fixed brain was consistent with the amount of PrP^{Sc} accumulation detected by PrP immunostaining (Fig. 2).

Finally, sCJD is primarily considered a disease of gray matter, although the extent of white matter involvement has not been well described. In the present study, PrP^{Sc} accumulation levels detected by PrP immunostaining in white matter were much lower than those in gray matter (Figs. 2a and 3). Many studies have reported that PrP^{Sc} is not seen or is very rarely seen in white matter (Parchi et al., 1996; Caverzasi et al., 2014). Some previous studies have reported the occasional presence of PrP^{Sc} in the white matter in sCJD (Muhleisen et al., 1995) and in mouse models, in which the PrP^{Sc} is not routinely detected in the white

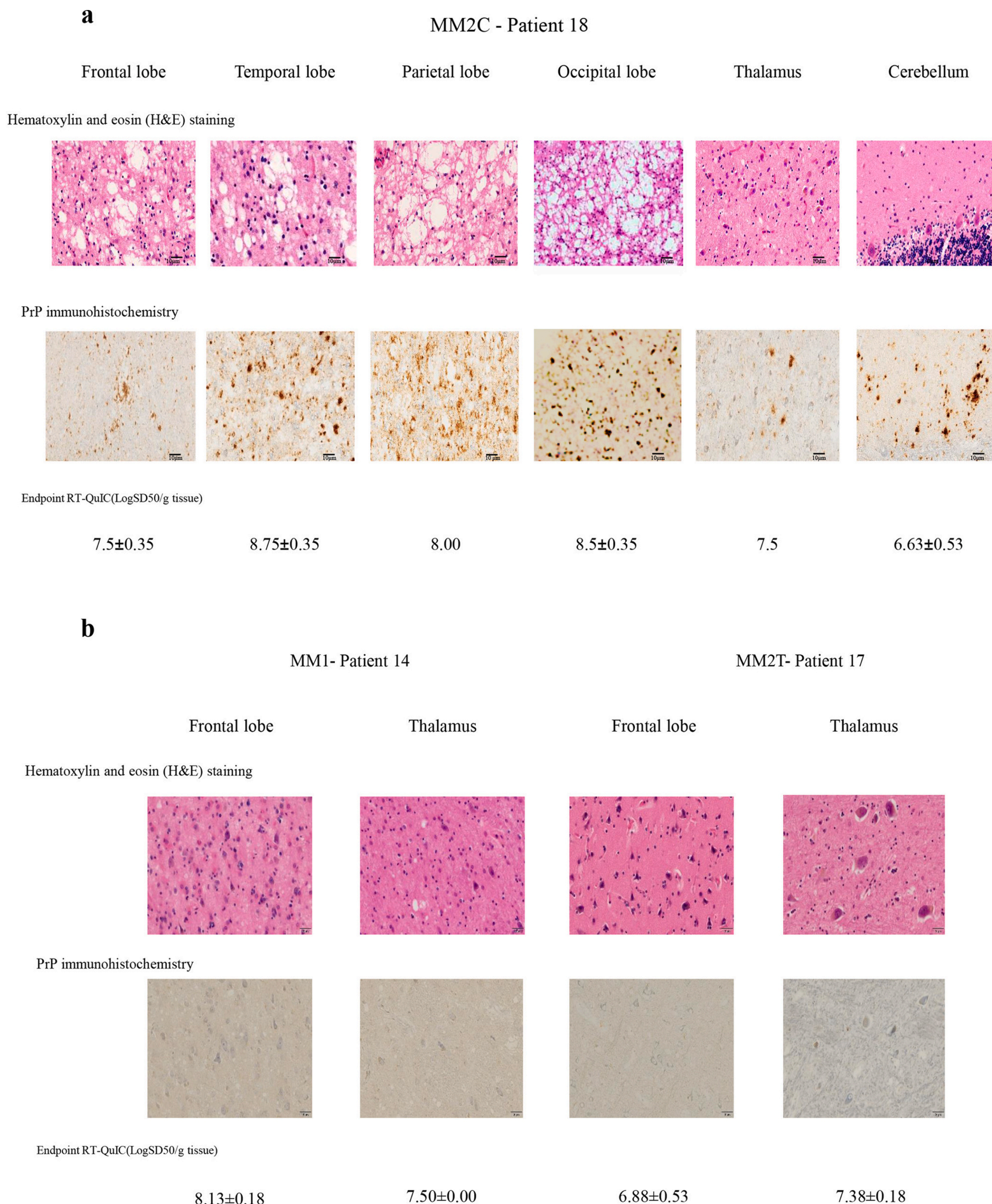

Fig. 2. Relationship between neuropathological findings and SD_{50} in sCJD patients.
(a) MM2-cortical form (patient 18): Immunostaining for PrP showed prominent perivacuolar-type and rough plaque-type (coarse-type) PrP deposits, whereas neuropathological examination revealed large confluent vacuole-type spongiform changes in the cerebral cortex. In the cerebral cortex, there was a relationship between the density of vacuoles and PrP deposition, as well as SD_{50}, except for in the frontal lobe. In addition, mild neurodegeneration and PrP deposits were observed in the thalamus and cerebellum.
(b) MM1 (patient 14): HE staining in the frontal lobe with high gliosis and high neuronal loss and synaptic-type PrP deposition in the frontal lobe by PrP immunostaining. Milder pathology was observed in the thalamus. MM2-thalamic form (patient 17): Mild spongy changes and very mild PrP deposition were observed in the thalamus and the frontal lobe by PrP immunostaining. Moreover, the log10 SD_{50} had a low value, ranging from 6.88–7.38 in the different regions examined.

Table 2
Distribution of prion seeding in six locations in sCJD brains.

Patient Number	MM1 (Patient 11)		MM1 (Patient 14)		MM2C (Patient 18)		MM2T (Patient 16)		MM2T (Patient 17)	
end-point RT-QuIC assay (logSD50)	Formalin-fixed brain (mean ±SD)	Formic acid-treated formalin-fixed brain (mean ±SD)	Formalin-fixed brain (mean ±SD)	Formic acid-treated formalin-fixed brain (mean ±SD)	Formalin-fixed brain (mean ±SD)	Formic acid-treated formalin-fixed brain (mean ±SD)	Formalin-fixed brain (mean ±SD)	Formic acid-treated formalin-fixed brain (mean ±SD)	Formalin-fixed brain (mean ±SD)	Formic acid-treated formalin-fixed brain (mean ±SD)
Frontal	7.75 ± 0.71	5.00 ± 0.00	8.13 ± 0.18	5.25 ± 0.00	7.5 ± 0.35	4.88 ± 0.17	5.88 ± 0.18	3.88 ± 0.18	6.88 ± 0.53	4.5 ± 0.35
Temporal	7.88 ± 0.18	4.62 ± 0.18	N.A.	N.A.	8.75 ± 0.35	5.88 ± 0.18	5.6 ± 0.18	3.63 ± 0.18	N.A.	N.A.
Parietal	7.88 ± 0.18	5.12 ± 0.18	N.A.	N.A.	8.00 ± 0.00	5.25 ± 0.00	5.75 ± 0.35	3.50 ± 0.00	N.A.	N.A.
Occipital	8.75 ± 0.35	5.75 ± 0.35	8.00 ± 0.35	4.88 ± 0.53	8.5 ± 0.35	5.25 ± 0.35	5.88 ± 0.18	3.75 ± 0.35	7.13 ± 0.18	4.75 ± 0.35
Thalamus	7.63 ± 0.53	4.63 ± 0.18	7.50 ± 0.00	4.88 ± 0.18	7.50 ± 0.00	4.25 ± 0.35	6.50 ± 0.35	4.13 ± 0.18	7.38 ± 0.18	4.88 ± 0.18
Cerebellum	8.88 ± 0.18	6.12 ± 0.18	8.38 ± 0.18	5.5 ± 0.35	6.63 ± 0.53	3.88 ± 0.53	6.63 ± 0.17	4.25 ± 0.35	7.00 ± 0.35	4.5 ± 0.35

N.A.: not available. Data are presented as means ± standard deviation.

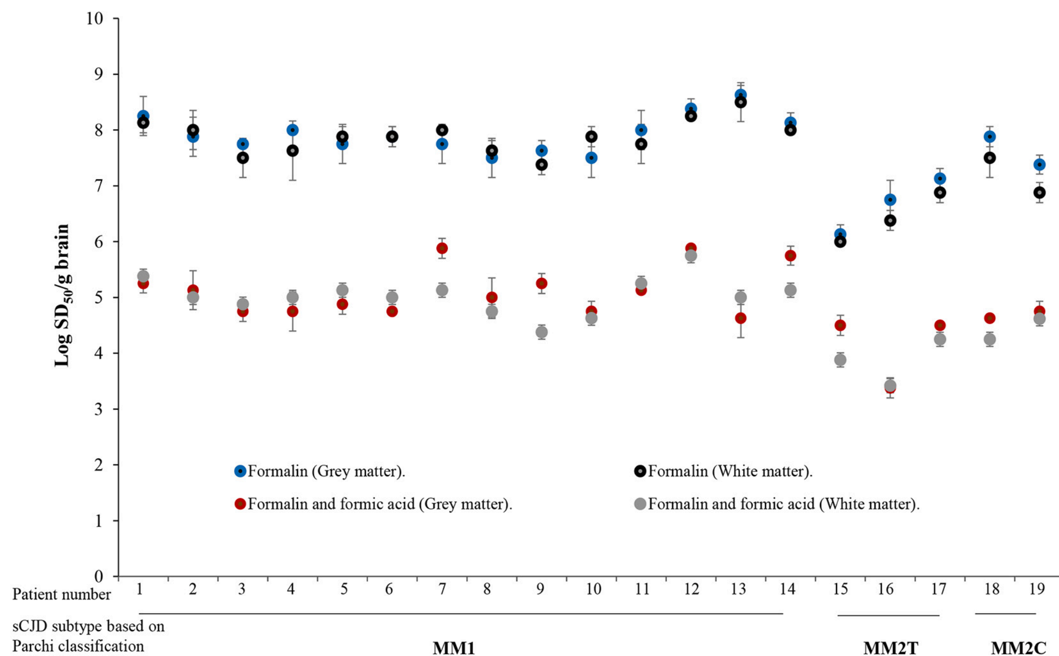

Fig. 3. Prion seeding in white and gray matter.
There was a slight difference between the SD_{50} in the white matter and gray matter in sCJD patients following formalin and formic acid treatment. The smallest difference was in the sCJD MM1 case, with a difference of 0.05 after formalin treatment, and 0.1 after combined formalin and formic acid treatment. In the MM2T group, the differences were 0.25 and 0.28, respectively. The largest differences were in the MM2C group, and were 0.44 and 0.25, respectively.

matter (DeArmond et al., 2002; Spilman et al., 2008), and suggest that it might be transported along axons to distant gray matter regions (Spilman et al., 2008). In the present study, we assessed the SD_{50} of white matter and gray matter treated with formalin and formic acid in sCJD patients and found a slight difference between the prion-seeding activity in the white matter and gray matter. These findings indicate that PrP^{Sc} accumulation was present in the gray matter, and that PrP^{Sc} accumulation was present in the white matter, despite not being apparent from pathological findings and PrP immunostaining.

5. Conclusion

We successfully used endpoint RT-QuIC for analysis of formalin-fixed brains of sCJD patients. We were able to detect SD_{50} in formalin-fixed brains of sCJD patients, and pathological analyses and quantitative biochemical methods can be performed after RT-QuIC. In the future, endpoint RT-QuIC with formalin-fixed sCJD brain samples will be a useful tool in combination with neuropathological analysis to improve understanding of sCJD.

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Consent for publication

We have obtained all authors' consent for publication.

Availability of data and material

Data sharing is not applicable to this article, as no datasets were generated during the current study.

Author contributions

Conceived and designed the experiments: K.S. and N.N. Performed the experiments: T.D. and T.Na. Analyzed the data: K.S., T.No., T.N., D. I., and N.N. Contributed reagents/materials/analytical tools and analyzed neuropathology: AA, B.M., M.T., and Y.I. Wrote the paper: K.S. and N.N.

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Credit author statement

Professor Katsuya Satoh, Corresponding author, is responsible for the credit associated with this manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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