Title: Potential for transmission of sporadic Creutzfeldt-Jakob disease through peripheral routes

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Abstract

Five sporadic Creutzfeldt-Jakob disease (CJD) strains have been identified to date, based on differences in clinicopathological features of the patients, the biochemical properties of abnormal prion proteins, and transmission properties. Recent advances in our knowledge about iatrogenic transmission of sporadic CJD have raised the possibility that the infectivity of sporadic CJD strains through peripheral routes is different from that of intracranial infection. To test this possibility, here we assessed systematically the infectivity of sporadic CJD strains through the peripheral route for the first time using a mouse model expressing human prion protein. Although the infectivity of the V2 and M1 sporadic CJD strains is almost the same in intracerebral transmission studies, the V2 strain infected more efficiently than the M1 strain through the peripheral route. The other sporadic CJD strains examined lacked infectivity. Of note, both the V2 and M1 strains showed preference for mice with the valine homozygosity at the *PRNP* polymorphic codon. These results indicate that the V2 strain is the most infectious sporadic CJD strain for infection through peripheral routes. In addition, these findings raise the possibility that individuals with the valine homozygosity at the *PRNP* polymorphic codon might have higher risks of infection through peripheral routes compared with the methionine homozygotes. Thus, preventive measures against the transmission of the V2 sporadic CJD strain will be important for the eradication of iatrogenic CJD transmission through peripheral routes.

Introduction

Creutzfeldt-Jakob disease (CJD) is a lethal transmissible neurodegenerative disease caused by an abnormal isoform of prion protein (PrP^{Sc}) converted from the normal cellular isoform (PrP^{C}) [1]. The conformational conversion of PrP^{C} can occur due to either one of three causes: spontaneous conversion in sporadic CJD (sCJD), pathogenic mutations of the *PRNP* gene in genetic CJD, or infection with PrP^{Sc} in iatrogenic CJD (iCJD) and variant CJD [2]. In iCJD, transmission of PrP^{Sc} from sCJD patients occurs through medical procedures such as dura mater grafting, intramuscular/subcutaneous administration of human growth hormone derived from cadaveric pituitary, corneal transplantation, neurosurgical operation, and electroencephalogram using deep brain electrodes [3].

Recent advances in our knowledge about transmission of sCJD have suggested that the infectivity of sporadic CJD strains through peripheral routes differs from that in intracranial infection [4]. sCJD is currently classified into six subtypes based on the genotype (methionine, M, or valine, V) at polymorphic codon 129 of the *PRNP* gene, the type (type 1, 21 kDa, or type 2, 19 kDa) of PrP^{Sc} accumulating in the brain, and neuropathologic features [5, 6]. The six sCJD subtypes have been proposed to harbor five sCJD strains: M1 (MM1/MV1 subtype corresponding to 40% of total sCJD patients), V2 (VV2 and MV2 subtypes, 23%), M2C (MM2C subtype, ≤ 1%), M2T (MM2T subtype, ≤ 1%), and V1 (VV1 subtype, ≤ 1%) [5-8]. In addition, co-occurrence of the M1 and M2C strains in the same patient has also been seen (MM1/MV1+2C subtype corresponding to 28% of total sCJD cases) [8]. Among the five sCJD strains, the M1 strain is the causative agent of the majority of dura mater graft-associated iCJD, reflecting the existing ratio of the sCJD subtypes in donors of the dura mater grafts [9, 10]. By contrast, in human growth hormone-associated iCJD, the M1 strain subgroup was underrepresented, and the V2 strain subgroup was overrepresented [11, 12]. In addition, the V2 strain subgroup was also predominant in kuru, which is caused by the transmission of sCJD

through ritual cannibalism [13, 14]. The reason for the different proportions of the causative sCJD strains among acquired prion diseases has remained elusive. Recently, we established a quick bioassay to evaluate the infectivity of PrP^{Sc} by intraperitoneal inoculation and found that a M1 sCJD isolate did not infect efficiently through the peripheral route [15]. This finding has raised the possibility that the predominance of the V2 strain subgroup in human growth hormone-associated iCJD and kuru might have been due to the weak infectivity of the M1 strain through peripheral routes. To test this possibility, here we performed a systematic analysis of the infectivity of sCJD strains through the peripheral route using multiple sCJD isolates.

Materials and methods

Transmission experiments

The sCJD cases included in this study were from patients with clinically, genetically and histopathologically proven sCJD, and brain tissues were obtained at autopsy from the patients after receiving written informed consent for research use. According to the current classification system of sCJD [5], these sCJD cases were classified as follows: VV2, 3 cases; MM1, 3 cases; MM2C, 3 cases; MM2T, 3 cases. Although two distinct MM2C subtypes harboring different prion strains have been recognized in Japan [16], the present study included the MM2C subtype showing large confluent vacuoles and perivacuolar PrP^{Sc} deposition. The production of knock-in mice expressing human PrP^{C} with the 129M/M genotype or 129V/V genotype (Ki-Hu129M/M or Ki-Hu129V/V) has been reported previously [17]. Brain homogenates (10%) were prepared in sterile phosphate-buffered saline using glass homogenizers, and 50 μl of the homogenates were intraperitoneally inoculated into Ki-Hu129M/M or Ki-Hu129V/V mice as described previously [18]. The inoculated mice were sacrificed at 75 days post-inoculation, and the spleen was immediately frozen for western

blotting. This bioassay system has been developed to evaluate rapidly the infectivity of PrP^{Sc} through the peripheral route [15, 17, 18].

Western blotting

Spleen tissues were homogenized in 2 ml of lysis buffer (100 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM $MgCl_2$, 2% Triton X-100, and 25 units/ml DNase I) and digested with collagenase (1 mg/200 mg tissue) overnight at room temperature. Collagenase digestion disrupts the connective tissue and improves the accessibility of detergents and/or proteinase K (PK) to PrP^{Sc}. The digested homogenates were ultracentrifuged at 323,000×*g* for 30 min at 4°C, and the pellets were resuspended and sonicated in 870 μl of PK-digestion buffer (100 mM Tris-HCl pH 8.0 and 5% Sarkosyl). The resuspended samples were centrifuged at 10,000×*g* for 3 min to remove the cell debris, and the supernatants (800 μl) were digested with PK (4 μg/200 mg tissue) for 1 h at 37°C. The PK-digested proteins were precipitated by adding 200 μl of 99.5% ethanol and ultracentrifugation at 100,000×*g* for 60 min at 4°C. The pellets were resuspended in Laemmli's sample buffer (60 mM Tris-HCl pH 6.8, 5% glycerol, 2% SDS, and 0.01% bromophenol blue) (400 μl/200 mg tissue). Protein samples were subjected to SDS-PAGE using 13.5% Tris-glycine long gels of 15 cm length and western blotting. Anti-PrP monoclonal antibody 3F4 (BioLegend, SanDiego, CA, USA) and type 2 PrP^{Sc}-specific polyclonal antibody Tohoku 2 [19] were used as the primary antibodies. Goat anti-mouse immunoglobulin polyclonal antibody labeled with a peroxidase-conjugated dextran polymer, EnVsion+ (Agilent, Santa Clara, CA, USA) and anti-rabbit EnVision+ (Agilent) were used as the secondary antibodies. The blots were visualized with Clarity Max Western ECL Substrate (Bio-Rad, Hercules, CA, USA), and images were obtained by imaging device ImageQuant LAS 4000 mini (GE Healthcare, Chicago, IL, USA).

Results

The V2 sCJD strain was efficiently transmitted to both Ki-Hu129M/M and Ki-Hu129V/V mice through the peripheral route. Total 58% of the intraperitoneally inoculated mice (39% in Ki-Hu129M/M and 74% in Ki-Hu129V/V) showed PrP^{Sc} accumulation in the spleen at 75 days post-inoculation (Table 1). The V2 strain-inoculated Ki-Hu129M/M mice produced the intermediate type PrP^{Sc} (20 kDa), while the V2 strain-inoculated Ki-Hu129V/V mice produced type 2 PrP^{Sc} (19 kDa) (Fig. 1), as reported previously [15, 20]. In contrast, the M1 sCJD strain was not efficiently transmitted to the human PrP^{C} knock-in mice by the peripheral route. One (H3) out of the three M1 isolates showed infectivity to both mouse lines [15], another isolate (I22) showed weak infectivity only to Ki-Hu129V/V mice, and the other isolate (J56) lacked infectivity. The total attack rate in the M1 strain-inoculated mice was 16% (6% in Ki-Hu129M/M and 26% in Ki-Hu129V/V). We have reported previously that the M1 isolate (H3)-inoculated Ki-Hu129M/M or Ki-Hu129V/V mice produced type 1 PrP^{Sc} (21 kDa) [15]. However, a single positive Ki-Hu129V/V mouse in the M1 isolate (I22)-inoculated group produced an unusual PrP^{Sc}, which migrated faster than the intermediate type PrP^{Sc} in the V2 isolate-inoculated Ki-Hu129M/M mice (Fig. 1). The M2C and M2T sCJD strains were not transmitted to both Ki-Hu129M/M and Ki-Hu129V/V mice by the peripheral route (Table 1).

Discussion

Here we investigated systematically the infectivity of sCJD strains through the peripheral route for the first time. The V2 strain showed stable infectivity, whereas the M1 strain showed only weak fluctuating infectivity. The M2C and M2T strains lacked infectivity. Of note, both the V2 and M1 strains showed relative preference for the mice with the 129V/V genotype. These

findings suggest that the V2 strain is the most infectious sCJD strain in infection through peripheral routes.

The infectivity of the M1 sCJD strain through the peripheral route was lower than that of the V2 sCJD strain. In intracerebral transmission studies, the M1 strain infected the human PrP^{C} knock-in mice as efficiently as the V2 strain [6, 19]. Therefore, peripheral tissue-specific factor(s) might reduce the infectivity of the M1 strain. Peripherally infected PrP^{Sc} is taken up by dendritic cells and is transported to lymphoid follicles [21, 22]. In lymphoid follicles, PrP^{Sc} is passed from dendritic cells to follicular dendritic cells and is replicated in follicular dendritic cells before invasion into the peripheral nervous system [23-25]. In this neuroinvasion process, the M1 PrP^{Sc} may have different properties compared to the V2 PrP^{Sc}, *e.g.*, stability in peripheral tissues, the efficiency of uptake by immune cells, or seeding activity for the replication in follicular dendritic cells. Recent reports suggest that spleen-derived PrP^{Sc} has more sialic acid on their *N*-linked glycans than brain-derived PrP^{Sc} [26], and that electrostatic repulsion between sialic acid molecules on *N*-linked glycans creates structural constraints controlling PrP^{Sc} replication rate [27]. Therefore, M1 PrP^{Sc} replication might be more affected by the sialylation of *N*-linked glycans than V2 PrP^{Sc} replication. Further studies will be needed in the future to unveil the reason for the reduced infectivity of the M1 strain through the peripheral route. On the other hand, in intracerebrally inoculated mice, the incubation period of the V2 strain was the shortest among the sCJD strains [6, 16, 19]. Therefore, the V2 strain might have the highest infectivity regardless of the route of infection. To address this point, further studies with quantitative infectivity titration will be needed.

The present study indicates that the underrepresentation of the M1 strain-originated subgroup and the overrepresentation of the V2 strain-originated subgroup in human growth hormone-associated iCJD may be due to the weak infectivity of the M1 strain through peripheral routes. It is noteworthy that the M2C strain did not infect through the peripheral

route in the present study, despite the finding that four out of twenty-one human growth hormone-associated iCJD patients showed neuropathologic features of the MM2C subtype besides VV2/MV2 features [12]. The infectivity of the M2C strain is extremely low even in intracerebral transmission experiments [6, 10], and transmission of the M2C strain has not been observed in dura mater graft-associated iCJD [10]. In addition, the duration of illness of the MM2C subtype is the longest among the sCJD subtypes [8]. Therefore, the replication of the M2C PrP^{Sc} might occur much less efficiently and require longer time, resulting in the lack of positive transmission in the present study. The reason for the discrepancy between the lack of positive transmission in the present study and the presence of the MM2C-like neuropathologic features in a part of human growth hormone-associated iCJD patients needs to be addressed in the future.

Unexpectedly, the M1 sCJD strain showed relative preference for the host with the 129V/V genotype in infection through the peripheral route. In intracerebral transmission experiments, the M1 and V2 strains are transmissible to both the 129M/M and 129V/V mice with almost 100% attack rates, but the incubation periods from inoculation to disease onset are prolonged in the codon 129 genotype-mismatched host [6, 19], suggesting that the homology of the codon 129 genotype between the inoculated sCJD strain and the host determines the efficiency of PrP^{Sc} replication. Indeed, the V2 strain showed preference for the host with the 129V/V genotype also in the present study. Although these findings raise the possibility that individuals with the 129V/V genotype might have higher potential risk of infection with CJD through peripheral routes compared with those with the 129M/M genotype, human growth hormone-associated iCJD case with the 129V/V genotype and MM1-like pathology or PrP^{Sc} type has not been recognized [11, 12]. In addition, the attack rates in the M1 strain-inoculated groups were variable, and the numbers of positive animals were very low in the present study. Furthermore, the present experimental design included neither an analysis of the spread of

PrP^{Sc} from the spleen to the brain nor other peripheral routes such as subcutaneous inoculation. Therefore, further studies with more M1 isolates or other routes of inoculation will be required in the future to conclude that individuals with the 129V/V genotype have higher potential risk of infection through peripheral routes. Furthermore, it will be of interest to investigate the pathology and PrP^{Sc} profiles in the brain of the intraperitoneally inoculated mice after disease onset. Particularly, in the present study, a single Ki-Hu129V/V mouse in the M1 isolate (I22)-inoculated group produced an unusual PrP^{Sc} migrating faster than the intermediate type PrP^{Sc}. This finding is not compatible with the data from the intracerebral transmission study of the M1 strain or those of the intraperitoneal transmission study of the other M1 isolate [6, 15, 20]. Therefore, it will be important to examine whether the change of PrP^{Sc} profiles could be reproduced in the brain of the Ki-Hu129V/V mice intraperitoneally inoculated with the M1 isolate (I22).

In conclusion, the present study shows that the V2 sCJD strain is more efficiently transmitted through the peripheral route than the M1 sCJD strain. Indeed, the V2 strain-originated subgroup has been reported not only in human growth hormone-associated iCJD patients but also in an occupational exposure-associated iCJD patient [28]. Since the current PrP^{Sc} disinfection/decontamination procedures had been developed using scrapie and CJD isolates before sCJD classification was established, the efficacy of these procedures against the V2 sCJD strain needs to be reconfirmed. In addition, as the presence of infectivity has been reported in a wide variety of peripheral tissues of sCJD MM1 patients including the lung, heart, kidney, pancreas, salivary gland, thyroid, adrenal gland, skeletal muscle, and lymphoid tissues [29], the distribution of infectivity in peripheral tissues should be investigated also in sCJD VV2 and MV2 patients. Furthermore, elucidation of the reason for the reduced infectivity of the M1 sCJD strain through the peripheral route will pave the way for the development of preventive measures against iatrogenic CJD transmission through peripheral routes. Finally, as

lymphoid tissues are more permissive than brain to cross-species transmission of chronic wasting disease and bovine spongiform encephalopathy [30], the bioassay system used in the present study will aid estimate the potentials for PrP^{Sc} infection through peripheral routes in animal-to-human transmission as well as human-to-human transmission.

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Conflict of interest

The authors report no competing interests.

Ethics approval

This study was approved by the Institutional Ethics Committee of Hokkaido University Faculty of Veterinary Medicine (approval number: VET1-5) and the Institutional Animal Care and Use Committees of Hokkaido University (approval number: 19-0025). All experiments using human materials were in compliance with the Helsinki Declaration. Animal experiments were performed in strict accordance with the Regulations for Animal Experiments and Related Activities at Hokkaido University.

Author contributions

Conceptualization: A. Kobayashi, T. Kitamoto; Methodology: S. Mohri, T. Kitamoto; Formal analysis and investigation: A. Kobayashi, Y. Munesue, T. Shimazaki; Writing - original draft preparation: A. Kobayashi; Writing - review and editing: K. Aoshima, T. Kimura, S. Mohri, T. Kitamoto; Funding acquisition: A. Kobayashi; Resources: T. Kitamoto, S. Mohri; Supervision: T. Kimura, S. Mohri, T. Kitamoto.

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Data availability

The data that support the findings are available from the corresponding author upon request.

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Figure legends

Fig. 1 Western blot analysis of protease-resistant PrP^{Sc} in the spleens of Ki-Hu129M/M or Ki-Hu129V/V mice intraperitoneally inoculated with sCJD isolates. An antibody reactive for all PrP^{Sc} types, 3F4, and a type 2 PrP^{Sc}-specific antibody, Tohoku 2 [19], were used for the western blotting. After challenge with the M1 isolate (I22), Ki-Hu129M/M mice lacked PrP^{Sc} accumulation in the spleen, while a single positive Ki-Hu129V/V mouse produced an unusual PrP^{Sc}, which migrated faster than the intermediate type PrP^{Sc} in the V2 isolate (AK)-inoculated Ki-Hu129M/M mice. The production of the intermediate type PrP^{Sc} in the V2 isolate (AK)-inoculated Ki-Hu129M/M mice and that of type 2 PrP^{Sc} in the V2 isolate (AK)-inoculated Ki-Hu129V/V mice have already been reported [20]. The M2C isolate (H93)- or the M2T isolate (J86)-inoculated mice lacked PrP^{Sc} accumulation in the spleen. MM, Ki-Hu129M/M mice. VV, Ki-Hu129V/V mice.

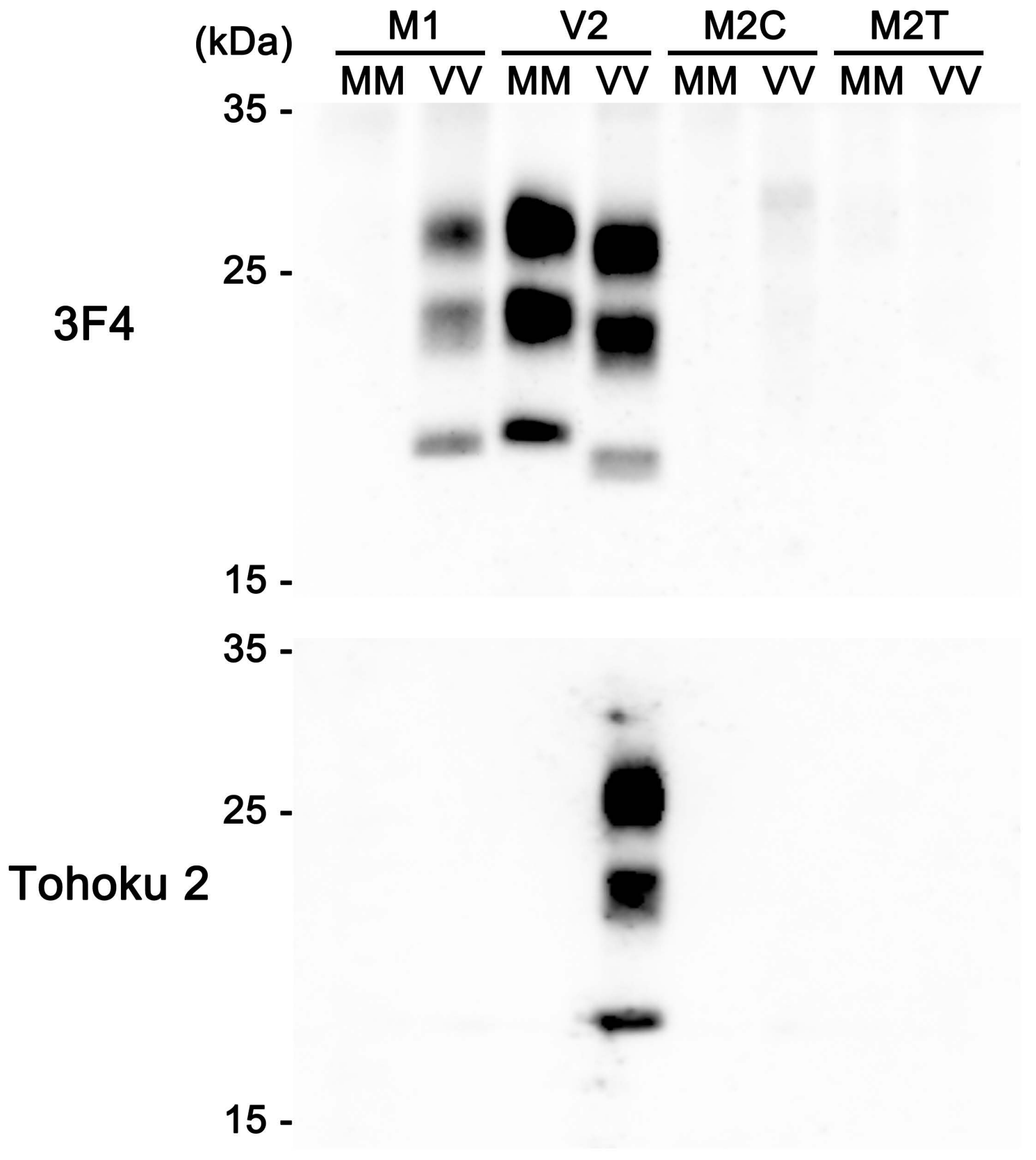
Table 1. Attack rates in the bioassay of sCJD strains

Inoculum		Attack rate n/n^0 [a] (%)		
sCJD strain	ID	Ki-Hu129M/M	Ki-Hu129V/V	Total
V2	J50	5/5 (100)	4/6 (67)	34/59 (58)
	AK[b]	3/10 (30)	10/12 (83)	
	AK[b] (re-evaluation)	2/7 (29)	5/6 (83)	
	J51[c]	1/6 (17)	4/7 (57)	
M1	J56	0/6 (0)	0/6 (0)	6/37 (16)
	I22	0/6 (0)	1/7 (14)	
	H3[c]	1/6 (17)	4/6 (67)	
M2C	J135	0/5 (0)	0/6 (0)	0/48 (0)
	H93	0/6 (0)	0/6 (0)	
	KZ[b]	0/6 (0)	0/7 (0)	
	KZ[b] (re-evaluation)	0/6 (0)	0/6 (0)	
M2T	J93	0/7 (0)	0/6 (0)	0/37 (0)
	J86	0/6 (0)	0/6 (0)	
	I110[c]	0/6 (0)	0/6 (0)	

[a] n/n^0, number of mice positive for PrP^{Sc} accumulation / number of inoculated mice.

[b] Bioassays of these isolates have been reported previously [15, 20], and the infectivity through the peripheral route was re-evaluated by the bioassay in the present study.

[c] Bioassays of these isolates have been reported previously [15].


(kDa)
M1
V2
M2C
M2T
MM VV MM VV MM VV MM VV
35 -
25 -
3F4
15 -
35 -
25 -
Tohoku 2
15 -