Application of protein misfolding cyclic amplification for the rapid diagnosis of acquired Creutzfeldt-Jakob disease

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Abstract

The susceptibility, clinical phenotype, and pathology of Creutzfeldt-Jakob disease (CJD) depends on both a methionine (M) /valine (V) polymorphism at codon 129 of the *PRNP* gene and the type of abnormal isoform of prion protein (PrP^{Sc}), either type 1 or type 2. The majority of CJD is sporadic CJD (sCJD) or genetic CJD, while CJD can be transmitted resulting in acquired CJD. It has been reported that dura mater graft-associated CJD (dCJD) with methionine homozygosity at codon 129 could be divided into distinct two phenotypes, namely the non-plaque-type of dCJD (np-dCJD) or the plaque-type of dCJD (p-dCJD). The cause of these two distinct phenotypes of dCJD was clarified by animal transmission studies using PrP-humanized mice based on the susceptibility and neurological or biochemical features in the mice inoculated with PrP^{Sc} from each dCJD subgroup. It is now likely that np-dCJD is associated with sCJD-MM1 or -MV1, the most common forms of sCJD (denoted as M1 strain in transmission studies), while p-dCJD is associated with sCJD-VV2 or -MV2, the second most common forms of sCJD (denoted as V2 strain). Although animal transmission studies are the most useful tool for identifying such atypical CJDs, relying exclusively on animal transmission studies may not be feasible due to the enormous cost and time. Therefore, we reported a method that can more easily and rapidly distinguish between M1-derived and V2-derived acquired CJDs using the protein misfolding cyclic amplification (PMCA) technique. Here, we describe the advantages of PMCA as a diagnostic tool for acquired CJD based on a comparison with those of conventional animal transmission studies.

1. Introduction

Creutzfeldt-Jakob disease (CJD) is a lethal neurodegenerative infectious disease. The key event in the pathogenesis of CJD is the conversion of normal cellular prion protein (PrP^{C}) into a disease-associated form of prion protein (PrP^{Sc}) (Prusiner et al. 1998). Although sporadic or genetic CJD is the most common form of CJD, CJD can also be acquired through infectious agents, namely prions, as in variant CJD (vCJD), kuru or iatrogenic CJD (iCJD). In acquired CJDs, iCJD is mainly caused by the transmission of prions via contaminated neurosurgical instruments, blood transfusion, cadaveric pituitary hormones, dura mater, or corneal grafts. The iCJD associated with the administration of contaminated human growth hormone from cadavers (hGH-CJD) and dura mater graft-associated CJD (dCJD) account for a large percentage of total iCJD cases (Brown et al. 2012). Worldwide, 226 cases of hGH-CJD and 228 cases of dCJD, including 142 cases in Japan, have been identified (Brown et al. 2006, Brown et al. 2012, Hoshi et al. 2000). It is notable that an atypical CJD showing distinct neuropathological features from those of classical CJD can be often seen in those with acquired CJD. Laboratory animal transmission studies are of great use for us to clarify the pathogenesis of atypical CJDs.

For example, vCJD is one of such acquired CJDs. After vCJD had first been reported in 1996 (Will et al. 1996), a total of 229 probable and definitive vCJD cases have been identified to date (Diack et al. 2014). Patients with vCJD show unusual clinical and pathological phenotypes such as early age at death and widespread deposition of a particular type of PrP plaque known as a florid plaque. After that, laboratory animal transmission studies using wild-type mice, PrP-humanized transgenic mice or macaque monkeys revealed that vCJD was a new form of CJD caused by cross-species transmission between cattle and humans via BSE prion-contaminated food distribution (Bruce et al. 1997, Lasmezas et al. 1996, Collinge et al. 1996).

The second example of acquired CJD showing unusual neuropathological appearances is plaque-type of dCJD (p-dCJD). The most common dCJD, non-plaque-type of dCJD (np-dCJD), shows clinicopathological features which cannot be distinguished from those of classical sCJD, whereas p-dCJD shows remarkable characteristics such as widespread amyloid plaques and an unusual type of PrP^{Sc} in the brain (Kretzschmar et al. 2003, Shimizu et al. 1999, Mochizuki et al. 2003). Because no case with characteristic features similar to those of p-dCJD had been reported among any sCJD subtypes, the cause of existence of two distinct dCJD subgroups had been a mystery. However, animal transmission studies with PrP-humanized knocked-in mice carrying human PrP with either the 129M/M or V/V genotype revealed that p-dCJD and np-dCJD were caused by infection with different PrP^{Sc} strains from distinct sCJD subgroups (Kobayashi et al. 2007, Kobayashi et al. 2009, Kobayashi et al. 2010).

Laboratory animal transmission studies have been the best way to identify new forms of human prion disease. Although prions newly generated through the infection of cross-species or intraspecies would cause unknown clinical and neurological appearances in hosts, the prions retain the inherent infectivity derived from their origins (Asano et al. 2006, Kobayashi et al. 2010). The origins of infection can be determined by transmission studies using laboratory animals based on the inherent infectivity. Therefore, the importance of animal transmission studies as a diagnostic tool to identify acquired CJD

will likely increase in the future for further improvement of CJD surveillance. Meanwhile, laboratory animal studies simultaneously have some problems which are difficult to resolve. A most serious issue is that animal transmission studies are extremely time-consuming. Moreover, it requires complicated work and is extremely costly. Not only the continuous effort to shorten the incubation time of animals, but also further efforts are needed to establish rapid and cheap alternatives to animal testing.

Therefore, we established a new, quick and reliable diagnostic method for acquired CJD cases using protein misfolding cyclic amplification (PMCA), in which prions can be amplified efficiently *in vitro* (Saborio et al. 2001). Using this diagnostic method, p-dCJD can be identified correctly within a week based on the amplification properties and the types of amplified products. Because no tissue or organ of healthy animals is needed but only cell-derived recombinant PrP^{C} as a PMCA substrate (denoted as cell-PMCA), our PMCA is a better method from the point of view of animal welfare. Here, we describe expectations and possibilities for the practical application of cell-PMCA as a tool for the diagnosis of human prion diseases through a comparison with conventional animal transmission studies.

2. Elucidation of cause of two distinct phenotypic features in dCJD

Basically, the clinical or biological heterogeneity of sCJDs is governed by both the genotype (methionine, M or valine, V) at the polymorphic codon 129 of the *PRNP* gene and the type (1 or 2) of PrP^{Sc} accumulating in the brain (Parchi et al. 1999). Type 1 and type 2 can be distinguished according to the size of the proteinase K-resistant core of the protein (21 kDa and 19 kDa, respectively) on western blots. Therefore sCJDs are classified into 6 subtypes (MM1, MM2, MV1, MV2, VV1 and VV2) (Parchi et al. 1999). Meanwhile, dCJD with codon 129 methionine homozygosity can be classified as different subtypes from those of sCJDs, namely, a non-plaque-type of dCJD (np-dCJD), a majority of the subgroup (68%) or plaque-type of dCJD (p-dCJD), and a minority of the subgroup (32%) (Yamada M, et al, 2009). The clinical and neuropathological features of np-dCJD cannot be differentiated from sCJD-MM1 or -MV1 and it shares phenotypic characteristics such as a diffuse synaptic PrP deposition with sCJD-MM1, or -MV1 patients (Hoshi K et al, 2000). However, p-dCJD shows distinct properties characterized by the presence of widespread kuru plaques and a unique PrP^{Sc} type with an electrophoretic mobility of about 20 kDa, which is intermediate in size between types 1 and 2 (Kobayashi et al. 2007). In order to clarify the cause of two distinct phenotypes in dCJD patients with the 129M/M genotype, we examined the transmission properties using humanized mice carrying human PrP with either the 129M/M or V/V genotype (Kobayashi et al. 2007, Kobayashi et al. 2009, Kobayashi et al. 2013, Kobayashi et al. 2014).

In these transmission studies, p-dCJD prions showed distinct transmissibility from that of np-dCJD prions. The neuropathological and biochemical features in the brains of the mice inoculated with p-dCJD prions were identical to those of sCJD-VV2 or -MV2 prions (denoted as V2 strain in transmission studies) (Bishop et al. 2010). In contrast, the transmission properties of np-dCJD prions were identical to those of sCJD-MM1 or -MV1 prions (denoted M1 strain in transmission studies) (Bishop et al. 2010). These results made clear that two distinct phenotypes in dCJD patients with the 129M/M genotype were caused by infection with the different PrP^{Sc} strains derived from distinct subtypes of sCJD, either the M1 or V2 strain. Thus, it was verified that p-dCJD is a

new form of acquired CJD caused by cross-sequential transmission of the V2 sCJD strain to individuals with the codon 129M/M genotype.

3. Application of cell-PMCA as a rapid diagnostic tool for acquired CJD

Our research on p-dCJD with animal transmission studies using PrP-humanized knocked-in mice so far revealed that the characteristics of both kuru plaques and the accumulation of a type i PrP^{Sc} among CJD patients with the 129M/M genotype (MMiK) can serve as diagnostic criteria for acquired CJD derived from the V2 strain (Kobayashi et al. 2014, Kobayashi et al. 2015). To avoid the potential risk of CJD transmission via unidentified routes, suspected cases need to be identified immediately by further review of archival materials, improved surveillance, careful analysis of the phenotypic features of the patients, and assessments of their infectivity. However, transmission studies are not appropriate for the screening of many specimens at a time due to the enormous cost and time. Therefore, to provide a rapid and accurate screening method for the assessment of infectivity in suspected cases, we adopted a PMCA technique for discriminating among V2-derived prions and M1-derived prions. (Takeuchi et al. 2016).

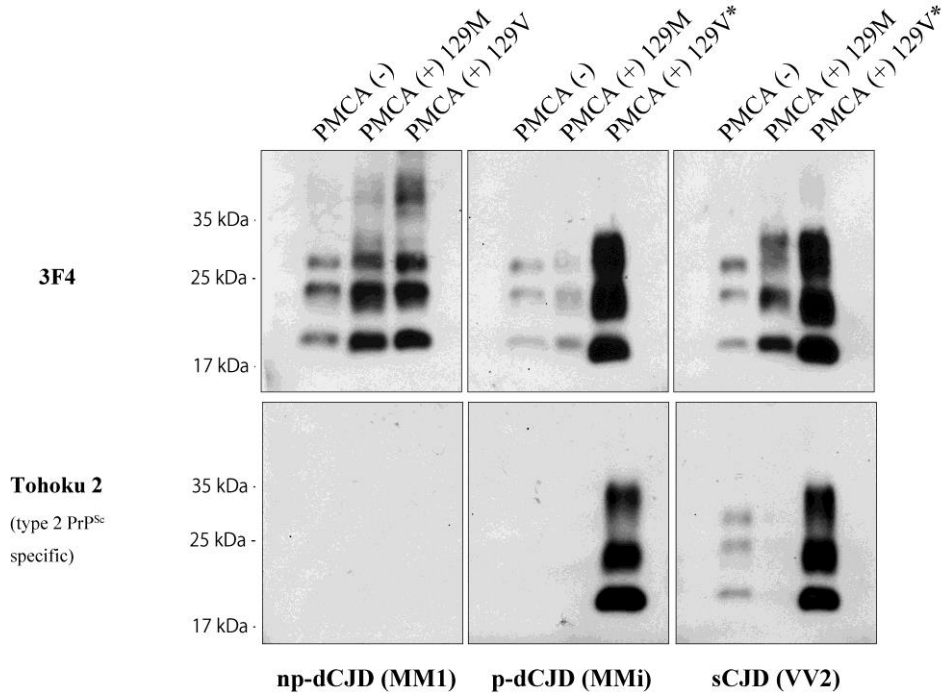

Figure 1. Seeding activity and types of amplified products of dCJD and sCJD prions with cell-PMCA. Ten% (w/v) np-dCJD (MM1), p-dCJD (MMi) and sCJD (VV2) brain homogenates were diluted in 20% (w/v) cell lysate derived from 293F cells (Invitrogen) stably expressing human PrP^{C} with the 129M or 129V genotype. Anti-PrP monoclonal antibody 3F4 (upper panel) or type 2 PrP^{Sc}-specific polyclonal antibody (Tohoku 2) (lower panel) was used as the primary antibody to detect PrP^{Sc}. The original PrP^{Sc} in p-dCJD is not recognized with Tohoku 2. Numbers indicate the molecular size standards (kDa). *Diluted (10-fold) sample was loaded because the signal was too strong.

PMCA is a technique for the amplification of PrP^{Sc} *in vitro* (Saborio et al. 2001). The propagation steps of PrP^{Sc} in PMCA are thought to mimic those *in vivo*, and the amplified products retain the biochemical features, structural properties or infectivity associated with the original PrP^{Sc} (Castilla et al. 2005, Castilla et al. 2008, Green et al. 2008). PMCA was developed as a method for ultra-high sensitive detection of animal prions such as hamster-adapted scrapie (Soto et al. 2005, Castilla et al. 2006), BSE (Murayama et al. 2014), chronic wasting disease (Kurt et al. 2007), or mouse-adapted scrapie (Murayama et al. 2007). In spite of the continuous effort to amplify human prions with PMCA to date, human prions, except for vCJD prions, have not been amplified as efficiently as animal prions (Yokoyama et al. 2011, Oshita et al. 2016, Belondrade et al. 2016). Our cell-PMCA using recombinant human PrP^{C} as a substrate can amplify sCJD-MV2 or -VV2 prions efficiently with cell lysates expressing human 129V PrP^{C} but not 129M PrP^{C} (Figure 1, upper panel). The amplified products could be detected by using a type 2 PrP^{Sc}-specific polyclonal antibody (Figure 1, lower panel). We expected that significant amplification would be demonstrated in p-dCJD prions with the 129V substrates if p-dCJD prions retain a similar seeding activity as that of sCJD-VV2 or -MV2 prions. As expected, the amplification properties of p-dCJD prions in cell-PMCA were identical to those of sCJD-MV2 or -VV2 prions (Figure 1, Table 1). In contrast, np-dCJD prions were not amplified so much even with 129M substrates of which the codon 129 genotype matched that of seeded np-dCJD prions (Figure 1, upper panel). Thus, cell-PMCA can distinguish p-dCJD from np-dCJD based on the clear preference of p-dCJD prions for the 129V genotype and the generation of type 2 PrP^{Sc}.

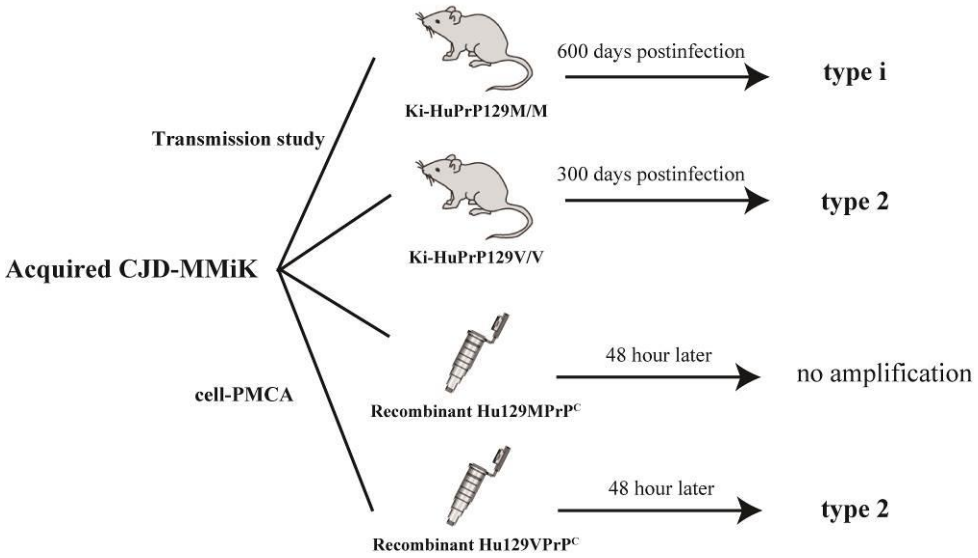

Figure 2. Schematic of the identification of acquired CJD-MMiK with animal transmission studies or cell-PMCA. Acquired CJD-MMiK prions are transmitted to PrP-humanized knocked-in mice carrying the 129M/M or V/V genotype, resulting in the generation of type i or type 2 PrP^{Sc} in the brains, respectively. In cell-PMCA, Mi prions are amplified efficiently only with the 129V substrates and produce type 2 PrP^{Sc}.

4. Comparison of conventional animal transmission study and cell-PMCA

To apply cell-PMCA as an alternative to animal transmission studies for identifying acquired CJD-MMiK cases, we compared the differences between cell-PMCA and conventional animal transmission studies in each diagnostic process and describe each advantage and disadvantage (Table 1 and Figure 2).

Table 1. Comparison of diagnostics for acquired CJD-MMiK with two different approaches

	Animal transmission study		cell-PMCA	
Host/ substrate	PrP-humanized knocked-in mice		Recombinant PrP^{C}	
PRNP codon 129 genotype	129M/M	129V/V	129M	129V
Incubation period (*days ±S.E. (n/n^0)*[a]) or PMCA efficiency (*relative amplification factor)*	Longer 685±51 (5/5)[b]	Short 259±6 (6/6)[b]	Poor >2 times [c]	Greater 50 times> [c]
Neulopathology	Amyloid plaques	Plaque like deposits	N/A	N/A
Type of generated PrP^{Sc}	Type i (20 kDa)	Type 2 (19 kDa)	N/A	Type 2 (in downshift)
Time required	over 2 years (About 600 days until disease onset in all of the animals)		1 week (PMCA for 48 hours)	
Cost	Expensive		Lower	

[a]n, number of diseased animals; n^0, number of inoculated animals.

[b]According to Kobayashi et al, 2014.

[c]Accoding to Takeuchi et al, 2016

[d]N/A, not applicable.

It is notable that the identification of acquired CJD-MMiK with cell-PMCA can be completed within a week because we can obtain the results with routine western blot analysis of the amplified products following proteinase K treatment after PMCA reaction for 48 hours. In contrast, in a transmission study, it takes more than 600 days until all the mice inoculated with p-dCJD prions develop the illness (Kobayashi et al. 2007, Kobayashi et al. 2014, Kobayashi et al. 2015). However, p-dCJD prions were successfully transmitted to both the 129M/M and V/V mice, and the specific neuropathological changes caused by Mi prions in the brain of the 129M/M mice, namely kuru plaques or a type i PrP^{Sc} accumulation, can raise the reliability of the diagnosis. In PMCA, it is impossible to recognize type i PrP^{Sc} in amplified products with the 129M substrate due to no or little amplification with the 129M substrate and the slight difference in the electrophoretic mobility of cell-derived PrP^{C} from that of brain-derived PrP^{C}.

The neuropathological information using laboratory animal transmission studies is invaluable to our understanding of the biology of acquired CJD-MMiK prions. However, humanized transgenic mouse expressing human PrP^{C} can be difficult to obtain, and a higher running cost is required in the management of laboratory animal experiments. In a situation where we work hard to reduce the use of animals in experiments, we confirmed the sufficient reliability for the identification of acquired CJD-MMiK with cell-PMCA. All 11 p-dCJD cases out of 114 CJD cases including sCJD, np-dCJD, p-dCJD or non-CJD were significantly amplified with 129V substrates and generated type 2 amplification products (Takeuchi et al. 2016). Neither misleading amplification nor the detection of type 2 amplification products with 129V substrates was observed among sCJD-MM1, -MM2C, -MM2T, np-dCJD, or non-CJD.

5. Remaining problems and future prospect of cell-PMCA

As described above, the discrimination between acquired CJD caused by the V2 strain and acquired CJD caused by the M1 strain can be carried out by cell-PMCA easily and very quickly with a high degree of reliability based on the amplification properties and the type of amplified products. However, when we apply cell-PMCA for the clinical diagnosis of CJD, there are still problems that need to be solved.

The absence of a report on the successful amplification of the whole spectrum of human prions prevents us from knowing whether the amplification of PrP^{Sc} in PMCA necessarily reflects the infectivity *in vivo*. We could not demonstrate that amplification of Mi prions with 129M substrates, whereas Mi prions were successfully transmitted to PrP-humanized mice carrying the 129M/M genotype as well as mice carrying the 129V/V genotype after a prolonged incubation period. We also did not observe an efficient amplification of M1 prions or an apparent preference for the genotype of the substrate, whereas its infectivity to laboratory animals has been fully demonstrated in previous studies. At present, highly efficient amplification using our cell-PMCA has been observed only in V2 or vCJD prions (Yokoyama et al. 2011, Takeuchi et al. 2016). Exponential amplification of M1 prions, the most common strain of sCJD, in a fully adapted reaction condition is the first step to overcome the discrepancy between PMCA and animal transmission studies. Therefore, we need to further optimize the reaction conditions to M1 prions. The amplification

efficiency of PrP^{Sc} is considerably affected by the substrate, such as normal human brain tissue, transgenic mice brains expressing human PrP^{C}, or recombinant PrP^{C} expressed in bacteria or cultured cells. The composition of the conversion buffer and the time or strength of the sonication are also important factors in the optimal amplification of PrP^{Sc}. Although the development of a fully adapted substrate or searching for the appropriate PMCA condition for the propagation of each prion strain *in vitro* would be exhausting work, these could lead to the standardization of PMCA in the future. Nevertheless, the great advantage of PMCA is that amplified products retain the biochemical properties of those of seeded PrP^{Sc} with a high degree of fidelity, unlike other cell-free conversion methods. The successful amplification of the whole spectrum of human prions would provide us not only with an ultra-high sensitive diagnostic method but also with a powerful tool for understanding the biology of human prions.

At present, cell-PMCA might be applied to another form of acquired CJD, hGH-CJDs, because it has been reported that hGH-CJD shares some neuropathological features with p-dCJD (Rudge et al. 2015, Cali et al. 2015). Recently, it was shown using some molecular approaches including PMCA technique that hGH-CJD cases in the UK resulted from transmission of the V2 strain (Ritchie et al. 2016). Although the number of patients with hGH-CJD has been decreasing, it is noteworthy that two patients with the 129M/M genotypes developed hGH-CJD almost 30 years after the first treatment in the UK (Ritchie et al. 2016). Therefore, hGH-CJD patients with the 129M/M genotype may possibly increase in the future after a longer incubation period caused by a genotypic barrier. In addition to enhancing surveillance, the application of cell-PMCA for many iCJD cases worldwide will enable us to identify the origins of infection more easily and rapidly.

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