nature medicine

Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion

Ryuichiro Atarashi[1,2], Katsuya Satoh[1], Kazunori Sano[1,3], Takayuki Fuse[1], Naohiro Yamaguchi[1], Daisuke Ishibashi[1], Takehiro Matsubara[1], Takehiro Nakagaki[1], Hitoki Yamanaka[4], Susumu Shirabe[5], Masahito Yamada[6], Hidehiro Mizusawa[7], Tetsuyuki Kitamoto[8], Genevieve Klug[9], Amelia McGlade[9], Steven J Collins[9] & Noriyuki Nishida[1,3]

The development of technologies for the *in vitro* amplification of abnormal conformations of prion protein (PrP^{Sc}) has generated the potential for sensitive detection of prions. Here we developed a new PrP^{Sc} amplification assay, called real-time quaking-induced conversion (RT-QUIC), which allows the detection of ≥1 fg of PrPSc in diluted Creutzfeldt-Jakob disease (CJD) brain homogenate. Moreover, we assessed the technique first in a series of Japanese subjects and then in a blind study of 30 cerebrospinal fluid specimens from Australia, which achieved greater than 80% sensitivity and 100% specificity. These findings indicate the promising enhanced diagnostic capacity of RT-QUIC in the antemortem evaluation of suspected CJD.

Transmissible spongiform encephalopathies, or prion diseases, are characteristically associated with the accumulation of PrP^{Sc} in the central nervous system through autocatalytic conversion of normal cellular PrP (PrP^{c}) into replicate misfolded isomers[1,2]. Despite a few other reported markers[3,4], PrP^{Sc} remains the best characterized and most reliable marker of prion disease.

Definitive antemortem confirmation of CJD requires the detection of PrP^{Sc} in biopsy specimens, the practice of which is discouraged because it is invasive and poses risks to health care personnel. Recently, however, *in vitro* PrP^{Sc} amplification techniques, including protein misfolding cyclic amplification[5–7], the amyloid seeding assay[8] and QUIC, have been reported to enable the direct and highly sensitive detection of PrP^{Sc} in various tissues, including cerebrospinal fluid (CSF). QUIC assays involve the use of soluble recombinant PrP (rPrP-sen) as a substrate, which is seeded with PrP^{Sc} and then subjected to intermittent automated shaking. This technique can be performed more easily than the protein misfolding cyclic amplification, which requires repeated sonication. Previous studies have shown that QUIC assays correctly discriminate between normal and scrapie-infected CSF samples in both hamster and sheep prion disease models[9,10]. However, ultrasensitive PrP^{Sc} detection in CSF from subjects with CJD has not yet been accomplished. Accordingly, we further refined the QUIC assay to improve its sensitivity and practicability and then applied the technique in a blind pilot study to detect PrP^{Sc} in CJD CSF specimens.

Given that a correlation between protease-resistant rPrP aggregate (rPrP-res) abundance and thioflavin T (ThT) fluorescence had been shown previously[7], we sought to determine the relative kinetics of rPrP-res formation by monitoring levels of ThT fluorescence in the QUIC assay. This was intended to minimize the time needed to detect rPrP-res. We first tested whether PrP^{Sc}-dependent rPrP-res ($rPrP\text{-}res^{Sc}$) formation could be induced in a microplate reader with intermittent shaking. We used human rPrP-sen (rHuPrP-sen) and a 10^{-7} dilution of CJD (molecular subtype MM1) brain homogenate as the substrate and seed, respectively. We conducted QUIC reactions at various concentrations (0, 0.25, 0.5 and 1.0 M) of guanidine-HCl (GdnHCl), because it has been shown that GdnHCl greatly enhances conversion of PrP-sen to PrP-res in cell-free conversion reactions[11]. Unexpectedly, we observed positive PrP^{Sc}-dependent ThT fluorescence within 24 h, both in the presence and in the absence of GdnHCl (**Fig. 1a**). In contrast, the negative control reactions without seed and in the absence of GdnHCl resulted in no increase in ThT fluorescence over 24 h; however, *de novo* formation of rPrP-res ($rPrP\text{-}res^{spon}$) was rapidly induced in the presence of GdnHCl when shaking was added (**Fig. 1a**,**b**). These results indicate that shaking accelerates PrP^{Sc}-dependent $rPrP\text{-}res^{Sc}$ formation even without GdnHCl (**Supplementary Fig. 1**), albeit with a lower peak of fluorescence.

Shaking is thought to cause partial unfolding of a portion of rPrP-sen by increasing the air-water interface[12]. Moreover, shaking enhances the interaction between rPrP-sen and PrP^{Sc} and promotes the fragmentation of rPrP-res polymers[13]. It is generally accepted that the energetic barrier of seed-dependent fibril formation and elongation is lower than that of spontaneous fibril formation, which first requires nucleation as the rate-limiting step[14]. The partial unfolding of rPrP-sen by shaking seems to be more heterogeneous than that facilitated by a denaturant such as GdnHCl, perhaps because the air-water interfaces created by shaking are unequally distributed

[1]Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan. [2]Nagasaki University Research Centre for Genomic Instability and Carcinogenesis, Nagasaki, Japan. [3]Global Centers of Excellence Program, Nagasaki University, Nagasaki, Japan. [4]Division of Comparative Medicine, Center for Frontier Life Sciences, Nagasaki University, Nagasaki, Japan. [5]Organization of Rural Medicine and Residency Education, Nagasaki University Hospital, Nagasaki, Japan. [6]Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan. [7]Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan. [8]Division of Creutzfeldt-Jakob Disease Science and Technology, Department of Prion Research, Tohoku University Graduate School of Medicine, Sendai, Japan. [9]Department of Pathology, Australian National Creutzfeldt-Jakob Disease Registry, University of Melbourne, Melbourne, Australia. Correspondence should be addressed to R.A. (atarashi@nagasaki-u.ac.jp).

Received 2 July 2010; accepted 16 December 2010; published online 30 January 2011; doi:10.1038/nm.2294

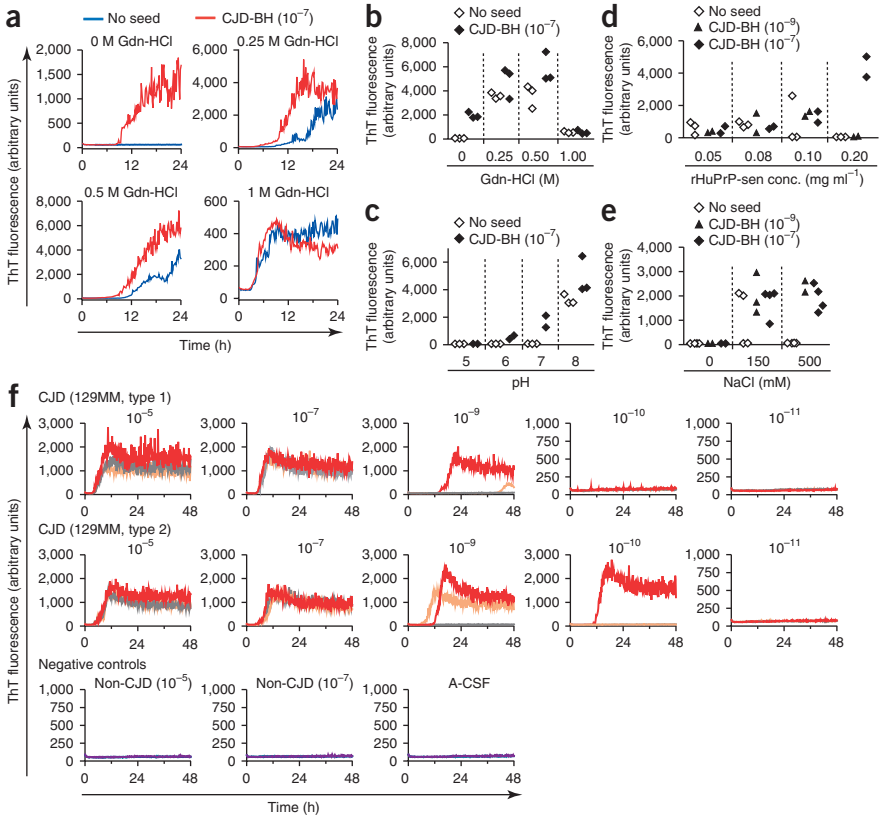

Figure 1 QUIC reactions induce PrP^{Sc}-dependent rHuPrP-res formation under GdnHCl-free conditions. (**a**,**b**) The effect of the indicated concentration of Gdn-HCl on the kinetics of rHuPrP fibril formation with or without 10^{-7} dilution of CJD brain homogenate (CJD-BH) (type 1, 129MM). The reaction buffer contained 150 mM NaCl, 50 mM PIPES, pH 7.0, 1 mM EDTA and 10 μM ThT. The concentration of rHuPrP-sen was 0.1 mg ml^{-1}. The graphs in **a** depict one representative of triplicates. The maximal fluorescence intensity of each single reaction for 24 h is plotted in **b**. (**c**–**e**) The effect of pH (**c**), the concentration of rHuPrP-sen (**d**) and the concentration of NaCl (**e**) were tested with the indicated dilutions of CJD-BH (type 1, 129MM) as seeds. Buffers used in **c** were pH 5 50 mM sodium acetate buffer, pH 6 50 mM MES, pH 7 50 mM PIPES and pH 8 50 mM HEPES. The concentration of NaCl in **c** and **d** was 150 mM, the pH of the buffer in **d** and **e** was 7.0 (50 mM PIPES) and the rHuPrP-sen concentration in **c** and **e** was 0.1 mg ml^{-1}. Each symbol represents the maximal fluorescence intensity from an individual reaction for 48 h. (**f**) Detection limit of RT-QUIC with the indicated dilutions of CJD-BH (129MM, type 1) and CJD-BH (129MM, type 2) as seeds. We used the indicated dilutions of non–CJD-BH (dissecting aneurysm) or artificial CSF (A-CSF) as negative controls. We performed the RT-QUIC reactions as described in the **Supplementary Methods**. The colored curves represent the kinetics of ThT fluorescence from an individual reaction seeded with the same dilution of BH. The graphs are representative of two independent experiments, each performed in triplicate.

in solution. We found that the addition of GdnHCl to QUIC reactions leads to an increase in the nucleation rate, and increased spontaneous fibril formation. The early appearance of rPrP-res^{spon} decreases the specificity of QUIC, because ThT fluorescence cannot distinguish between rPrP-res^{Sc} and rPrP-res^{spon}. Therefore, we chose not to use GdnHCl in subsequent analyses.

To further optimize the conditions, we examined the effects of pH, as well as of the concentrations of rHuPrP-sen and salt on QUIC reactions in GdnHCl-free conditions with shaking (**Fig. 1c–e** and **Supplementary Fig. 2**). After assessment, we successfully established a method for the real-time monitoring of the kinetics of rPrP fibril formation seeded with CJD brain homogenate (see **Supplementary Methods**), without the generation of rPrP-res^{spon} and designated the assay RT-QUIC.

To determine the minimum amount of PrP^{Sc} detectable by RT-QUIC, we diluted CJD brain homogenate (MM1 and MM2) serially with artificial CSF (A-CSF) and used these dilutions to seed the reactions. We observed increased PrP^{Sc}-dependent ThT fluorescence within 48 h in more than half the replicates of CJD brain homogenate, with dilutions ranging from 10^{-5} to 10^{-9} (**Fig. 1f** and **Supplementary Table 1**). With 10^{-10} brain homogenate dilutions, we observed a marginally lower rate of positive reactions, and 10^{-11} dilutions of the CJD-brain homogenates produced no ThT fluorescence response (**Fig. 1f** and **Supplementary Table 1**). The negative controls seeded with 10^{-5} and 10^{-7} dilutions of non-CJD brain homogenate or A-CSF alone (no seed) did not produce an increase in the fluorescence (**Fig. 1f** and **Supplementary Table 1**). The 10^{-9} dilutions of MM1 and MM2 CJD brain homogenate contained approximately 0.8 and 1.9 fg of PrP^{Sc}, respectively, according to our estimation (data not shown). Consequently, the results indicate that this assay consistently enables us to detect more than or equal to about 1 fg of PrP^{Sc} in the diluted CJD brain homogenates within 48 h. Moreover, the fact that there was no rPrP-res^{spon} formation under the conditions used implies a lower and acceptable risk of false-positive reactions. Whether the RT-QUIC has the same sensitivity to CJD brain homogenate with 129MV or 129VV as 129MM remains to be determined.

CSF is routinely used in the evaluation of central nervous system disorders and presumably contains more PrP^{Sc} and fewer impurities than blood. This prompted us to compare the RT-QUIC seeding activity in CSF samples from subjects with CJD and subjects without CJD but with other neurodegenerative diseases. For the pilot study, we initially tested CJD CSF samples from 18 definite cases of CJD in Japan (**Table 1**) and 35 non-CJD controls from subjects with other neurodegenerative diseases (**Supplementary Table 2**). We saw a minimal ThT fluorescence increase in the controls, with no false positives in the assay. In contrast, increased PrP^{Sc}-dependent fluorescence was seen in at least one of four replicates in 15/18 (83.3%) of the CJD CSF samples (**Table 1** and **Supplementary Table 3**).

To further confirm the reliability of RT-QUIC, we conducted a blind trial using 30 CSF samples from the Australian National CJD Registry and 155 CSF samples containing 25 probable cases of CJD and 130 cases of other neurological diseases obtained in Japan. In the Australian samples, we were able to detect PrP^{Sc} in 14/16 (87.5%) definite CJD CSF samples, as opposed to 0/14 of the non-CJD controls (**Table 1**, **Supplementary Fig. 3** and **Supplementary Table 3**). It should be noted that 3/4 129VV and 2/2 129MV cases were positive by RT-QUIC.

Table 1 Clinical data and RT-QUIC reactions seeded with CSF samples

CSF from 18 definite CJD samples in Japan					
Sample	Age (years)/sex	CJD type	Molecular subtype[a]	14-3-3 (γ-isoform)[b]	RT- QUIC[c]
C1	68/M	sCJD	MM 1	+	+ (3/4)
C2	66/F	sCJD	MM 1	+	+ (2/4)
C3	71/F	sCJD	MM 1	+	+ (1/4)
C4	57/F	sCJD	MM 2	+	+ (2/4)
C5	70/M	sCJD	MM 2	+	+ (2/4)
C6	66/M	sCJD	MM 2	+	+ (2/4)
C7	60/F	sCJD	MM 2	–	– (0/4)
C8	73/F	sCJD	MM 2	+	+ (4/4)
C9	74/M	sCJD	MM 2	+	– (0/4)
C10	79/F	sCJD	MM 2	–	+ (3/4)
C11	65/F	sCJD	MM 2	+	+ (4/4)
C12	69/M	sCJD	MM 2	+	– (0/4)
C13	69/F	sCJD	MM 2	+	+ (3/4)
C14	54/F	sCJD	MM 2	+	+ (3/4)
C15	76/F	sCJD	MM 2	–	+ (2/4)
C16	68/M	sCJD	MM 2	–	+ (4/4)
C17	58/F	iCJD	MM 1	+	+ (3/4)
C18	79/F	iCJD	MM 1	–	+ (3/4)

Blind trial of 30 CSF samples in Australia						
Sample	Age (years)/sex	Diagnosis[d]	Codon 129	Profile of PrP^{Sc}	14-3-3 (all isoforms)[e]	RT-QUIC[g]
A1	53/F	PN/MC			–	– (0/4)
A2	59/F	PN/ MC			–	– (0/4)
A3	85/M	AD			–	– (0/4)
A4	60/F	ICD			+	– (0/4)
A5	83/M	AD			+	– (0/4)
A6	73/M	sCJD	VV	2	+	+ (2/4)
A7	67/F	sCJD	MM	1	+	+ (4/4)
A8	82/F	sCJD	MM	1	+	+ (2/4)
A9	67/M	sCJD	MV	1	–	+ (2/4)
A10	50/M	sCJD	MM	1	+	+ (3/4)
A11	66/M	PN/MC			–	– (0/4)
A12	61/M	PN/MC			–	– (0/4)
A13	84/F	sCJD	MM	1	+	+ (3/4)
A14	76/M	sCJD	MM	1	+	+ (2/4)
A15	69/M	sCJD	MV	1	+	+ (4/4)
A16	67/M	AD			–	– (0/4)
A17	75/F	PN/MC			+	– (0/4)
A18	93/M	DLB/PD			–	– (0/4)
A19	67/F	sCJD	ND	2	+	+ (2/4)
A20	53/M	DLB/PD			–	– (0/4)
A21	71/F	sCJD	VV	2	+	– (0/4)
A22	62/F	sCJD	MM	2	–	– (0/4)
A23	90/M	sCJD	VV	ND	+	+ (1/4)
A24	61/F	DLB/PD			–	– (0/4)
A25	74/M	sCJD	MM	ND	±[f]	+ (2/4)
A26	74/F	AD			–	– (0/4)
A27	68/F	sCJD	ND	ND	+	+ (2/4)
A28	69/F	sCJD	VV	2	+	+ (1/4)
A29	82/M	DLB/PD			+	– (0/4)
A30	70/F	sCJD	ND	2	+	+ (4/4)

[a]CJD can be divided into six molecular subtypes on the basis of whether methionine (M) or valine (V) is present at codon 129 of the gene encoding prion protein combined with the profile of PrP^{Sc} (type 1 or type 2) as determined by western blotting[17]. [b]Amounts of the γ-isoform of 14-3-3 protein in CSF were determined by western blotting with a polyclonal antibody specific for the γ-isoform of the 14-3-3 protein[18] (+, positive reaction; –, negative reaction). [c]RT-QUIC was performed as described in the **Supplementary Methods**. The number of positive reactions over the number of replicates is shown in parentheses. Samples with at least one positive reaction were defined as +, representing a positive result in the RT-QUIC. [d]The final diagnosis was made by the Australian National CJD Registry. [e]Amounts of 14-3-3 protein (all isoforms) in CSF were determined by western blotting. [f]Additional atypical bands were observed. [g]Kinetics graphs are provided in **Supplementary Figure 3**. Subjects or their families agreed with the aims and role of our research and gave appropriate informed consent. The investigation protocol 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). sCJD, sporadic Creutzfeldt-Jakob disease; iCJD, iatrogenic Creutzfeldt-Jakob disease; PN/MC, paraneoplastic/metastatic cancer; ICD, inflammatory central nervous system disorder; DLB/PD, dementia with Lewy bodies/Parkinson's disease; AD, Alzheimer's disease; ND, not determined.

These results indicate that RT-QUIC has the ability to discriminate CJD CSF samples that include 129MM, 129MV and 129VV cases from non-CJD CSF samples. In addition, none of the 130 Japanese cases of other neurological diseases were positive, further confirming the reliability of this assay (**Supplementary Table 4**). Collectively, the RT-QUIC assays showed more than 80% sensitivity and 100% specificity. The sensitivity was equivalent to and the specificity was much higher than that achieved by 14-3-3 (refs. 14,15), a nonspecific marker of rapid neuronal damage (**Supplementary Table 3**).

Although we have never experienced a false-positive reaction among the hundreds of non-CJD neurodegenerative disease samples we have so far tested, it remains possible that certain conditions may evoke a positive reaction, and further studies will be required to eliminate this possibility. Furthermore, scrupulous attention to the conditions of the assay is essential to avoid false positives in the clinical setting. Nevertheless, we believe that the ultrasensitive detection of PrP^{Sc} in CSF by RT-QUIC represents a valuable new means for the early, rapid and specific diagnosis of CJD.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank the members of the CJD Surveillance Committee in Japan for their support of this work, K. Yamaguchi and K. Kuwata for helping with the circular dichroism analysis of rPrP-sen, M. Tsujihata (Nagasaki Kita Hospital) for providing CSF samples and information about subjects and A. Yamakawa and A. Matsuo for technical assistance. The work was supported by the Global Centers of Excellence Program (F12); a grant-in-aid for science research (B; grant no. 20390287) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant for bovine spongiform encephalopathy research and a grant-in-aid of the Research Committee of Prion disease and Slow Virus Infection, from the Ministry of Health, Labor and Welfare of Japan.

AUTHOR CONTRIBUTIONS

R.A. designed the project, performed experiments and wrote the manuscript. K. Satoh, K. Sano, T.F., N.Y., D.I., T.M., T.N. and H.Y. performed experiments. K. Satoh, S.S., M.Y., H.M., T.K., G.K., A.M. and S.J.C. contributed to the collection of human specimens and provided information about subjects. N.N. supervised the project. K. Satoh, K. Sano, A.M., S.J.C. and N.N. helped with the editing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemedicine/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Prusiner, S.B. *Proc. Natl. Acad. Sci. USA* **95**, 13363–13383 (1998).
2. Aguzzi, A. & Calella, A.M. *Physiol. Rev.* **89**, 1105–1152 (2009).
3. Lasmezas, C.I. *et al. Science* **275**, 402–405 (1997).
4. Gambetti, P. *et al. Ann. Neurol.* **63**, 697–708 (2008).
5. Saborio, G.P., Permanne, B. & Soto, C. *Nature* **411**, 810–813 (2001).
6. Castilla, J. *et al. Methods Enzymol.* **412**, 3–21 (2006).
7. Atarashi, R. *et al. Nat. Methods* **4**, 645–650 (2007).
8. Colby, D.W. *et al. Proc. Natl. Acad. Sci. USA* **104**, 20914–20919 (2007).
9. Atarashi, R. *et al. Nat. Methods* **5**, 211–212 (2008).
10. Orru, C.D. *et al. Protein Eng. Des. Sel.* **22**, 515–521 (2009).
11. Kocisko, D.A. *et al. Nature* **370**, 471–474 (1994).
12. Toth, S.I., Smith, L.A. & Ahmed, S.A. *J. Pharm. Sci.* **98**, 3302–3311 (2009).
13. Collins, S.R., Douglass, A., Vale, R.D. & Weissman, J.S. *PLoS Biol.* **2**, e321 (2004).
14. Lansbury, P.T. Jr. & Caughey, B. *Chem. Biol.* **2**, 1–5 (1995).
15. Hsich, G., Kenney, K., Gibbs, C.J., Lee, K.H. & Harrington, M.G. *N. Engl. J. Med.* **335**, 924–930 (1996).
16. Zerr, I. *et al. Ann. Neurol.* **43**, 32–40 (1998).
17. Parchi, P. *et al. Ann. Neurol.* **46**, 224–233 (1999).
18. Satoh, K. *et al. Lab. Invest.* **90**, 1637–1644 (2010).