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Real-time quaking-induced conversion

A highly sensitive assay for prion detection

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We recently developed a new in vitro amplification technology, designated "real-time quaking-induced conversion (RT-QUIC)," for detection of the abnormal form of prion protein (PrP^{Sc}) in easily accessible specimens such as cerebrospinal fluid (CSF). After assessment of more than 200 CSF specimens from Japanese and Australian patients, we found no instance of a false positive, and more than 80% accuracy for the correct diagnosis of sporadic Creutzfeldt-Jakob disease (sCJD). Furthermore, the RT-QUIC can be applied to other prion diseases, including scrapie, chronic wasting disease (CWD) and bovine spongiform encephalopathy (BSE), and is able to quantify prion seeding activity when combined with an end-point dilution of samples. These results indicate that the RT-QUIC, with its high sensitivity and specificity, will be of great use as an early, rapid and specific assay for prion diseases.

Key words: RT-QUIC, real-time quaking-induced conversion, prion, CJD, Creutzfeldt-Jakob disease, CSF, cerebrospinal fluid

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Diagnosis of Creutzfeldt-Jakob Disease: The Current Situation

Human prion diseases, including Creutzfeldt-Jakob disease (CJD), are incurable neurodegenerative disorders characterized by progressive spongiform changes and the accumulation of abnormal prion protein (PrP^{Sc}) in the central nervous system.[1] The majority of CJD cases (approximately 85%) are sporadic in nature, but the remaining cases comprise genetic and infectious forms. Iatrogenic CJD is the consequence of inadvertent transmission during medical procedures in which sporadic CJD (sCJD)-contaminated tissues or explants (such as dura mater and pituitary hormones) or surgical instruments were used.[2] Variant CJD (vCJD) is primarily a zoonosis that arose from contamination of the human food chain by bovine spongiform encephalopathy (BSE), although secondary transmission of vCJD by blood transfusion has also been reported in reference 3. Hence, by adopting additional infection control measures as appropriate, an early and accurate diagnosis of CJD would help to lessen the possibility of iatrogenic transmission and lead the way to timely therapeutic interventions. However, the definitive ante-mortem confirmation of CJD currently requires the presence of typical neuropathology together with the demonstration of PrP^{Sc} in specimens obtained by biopsy, the practice of which is often precluded both by the invasiveness of the procedure and the risks it poses to medical care staff. Thus, the highly sensitive detection of PrP^{Sc} in accessible body fluids such as cerebrospinal fluid (CSF) and blood can be expected to constitute a most valuable means for the early and specific diagnosis of CJD. However, because the concentration of PrP^{Sc} in these specimens is likely to be very low, one of the most promising approaches would be to develop an efficient amplification of PrP^{Sc} in vitro.[4,5] Indeed, several assays, including protein misfolding cyclic amplification (PMCA),[6,7] the amyloid seeding assay (ASA),[8] and quaking-induced conversion (QUIC),[9,10] have previously been reported to permit the sensitive detection of PrP^{Sc} in animal and human brain specimens. Nonetheless, early attempts at

ultrasensitive PrP^{Sc} detection in accessible body fluids were unsuccessful in human prion diseases. For this reason, we initiated studies aimed at establishing a highly sensitive assay for the detection of prion in human CSF.

Establishment of Real-Time QUIC (RT-QUIC)

In the QUIC assays, soluble recombinant PrP (rPrP-sen) expressed in *E. coli* is used as a substrate to amplify the minute amounts of PrP^{Sc}. Using a dedicated shaker, the reaction is enhanced by vigorous intermittent shaking which induces rPrP-sen to aggregate and form fibrils.[9] One of the advantages in QUIC is that shaking/agitation can be performed more easily and consistently than sonication, which has the problem of varied delivery of vibrational energy to the samples. On the other hand, further improvement in the rapidity and practicality of this method was required in order for it to become useful in the diagnosis of prion diseases, because the initial standard format of QUIC (S-QUIC) required a time-consuming western blot. Thus, we combined QUIC technology with thioflavin T (ThT) fluorescence dye, to monitor amyloid formation, in order to minimize the time necessary for the detection of protease-resistant rPrP fibrils (rPrP-res). We first determined if the shaking of our fluorescence microplate reader could induce PrP^{Sc}-dependent rPrP-res ($rPrP\text{-}res^{Sc}$) formation in a buffer containing 0.05–0.1% SDS, as in the S-QUIC. Human rPrP-sen (rHuPrP-sen) and CJD brain homogenate (BH) were used as a substrate and a seed, respectively, for the QUIC reaction. Unexpectedly, we did not observe $rPrP\text{-}res^{Sc}$ formation or an elevation in the ThT fluorescence using our microplate reader (unpublished data). Our explanation for this is that the shaking power of the microplate reader was not strong enough to elicit the QUIC reactions in the presence of SDS. SDS tends to cause fibrils to stack and stabilize rPrP-res polymers as a result. In fact, we observed that fibrils formed in the presence of SDS were much larger and thicker than those formed in its absence. Taken together, sonication in PMCA or vigorous shaking in S-QUIC seems to be required as a means of fragmenting the rPrP-res polymers formed in the presence of SDS.

We then tested whether $rPrP\text{-}res^{Sc}$ formation was induced when guanidine-HCl (GdnHCl) was added, because it has been thought that GdnHCl was required for the conversion of PrP-sen to PrP-res in a cell-free system.[11] Somewhat unexpectedly, we observed $rPrP\text{-}res^{Sc}$ formation even in the absence of GdnHCl. In contrast, the negative control reactions without seed and in the absence of GdnHCl exhibited a marked delay in spontaneous rPrP-res ($rPrP\text{-}res^{spon}$) formation.[12] For this reason, use of a GdnHCl-free buffer can dramatically reduce the risk of false-positive reactions and enhance the sensitivity of the method.

Shaking/agitation is considered to cause several facilitatory effects on the QUIC reaction. One is that a partial unfolding of a portion of the rPrP-sen is induced by increasing the air-water interface through which a denaturing boundary between the hydrophobic air and hydrophilic water is formed.[13] Next, shaking/agitation enhances the interaction between rPrP-sen and PrP^{Sc}, and the fragmentation of rPrP-res polymers.[14] The energetic barrier of spontaneous fibril formation is likely to be higher than that of seed-dependent fibril formation or elongation, because spontaneous formation initially necessitates nucleation as the rate-limiting step.[15]

Meanwhile, the extent of the partial unfolding of rPrP-sen by shaking alone is assumed to be more heterogeneous than that in the presence of GdnHCl, probably because the air-water interfaces are unequally distributed in the reaction mixture (**Fig. 1**). In contrast, the addition of GdnHCl accelerates the nucleation rate, resulting in an increase in the rate of spontaneous fibril formation. Of note, we observed that there was an inverse correlation between the rate of rPrP-res formation and the concentration of rPrP-sen substrate.[12] It has been reported that the aggregation rate of several other proteins is inversely correlated with the concentration of substrate protein in a denaturant-free buffer with shaking.[16,17] Conversely, previous studies using cell-free conversion[18] and rPrP fibril formation,[19-21] respectively, in the presence of denaturant or at low pH, have shown that the rate of PrP-res formation was directly proportional to the PrP-sen concentration. This seeming contradiction can be explained again by the difference in the denaturation status of PrP-sen under various conditions. We hypothesized that heterogenous denaturation of the substrate protein in a denaturant-free buffer with shaking is a major cause of the inverse correlation (**Fig. 1**).

We examined the effect of pH, and the concentrations of rHuPrP-sen and salt, on QUIC reactions in a GdnHCl-free buffer. We found that the presence of NaCl is essential for rPrP-res formation and the sensitivity of this method was maximal at 500 mM NaCl at pH 7.[12] The requirement for NaCl in the formation of rPrP-res is compatible with previous studies, which have shown that salt is required for cell-free conversion in the absence of GdnHCl[22] and the maintenance of a protease-resistant PrP^{Sc} conformation.[23]

We named this new assay "real-time QUIC (RT-QUIC)" by analogy with real-time PCR. The RT-QUIC enabled us to measure up to 96 replicates at a time, obtain the results immediately, and is potentially safer than S-QUIC or PMCA because the prions are sealed within a 96-well plate throughout the entire procedure.

Application of RT-QUIC to Diagnostic Tests for Human Prion Diseases

CJD has been categorized into six molecular subtypes (MM1, MM2, MV1, MV2, VV1, VV2) 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).[24] We evaluated the detection limit of MM1- and MM2-sCJD brain homogenate using RT-QUIC. The minimum amount of PrP^{Sc} in the brains detectable by RT-QUIC was around ~1 fg (10^{-15} g). To determine the applicability of RT-QUIC in the clinical diagnosis of sCJD, we compared the RT-QUIC seeding activity in CSF samples from patients with sCJD and patients without sCJD but with other neurodegenerative diseases such as Alzheimer disease. We decided upon CSF as the specimen because CSF is routinely used in the assessment of many

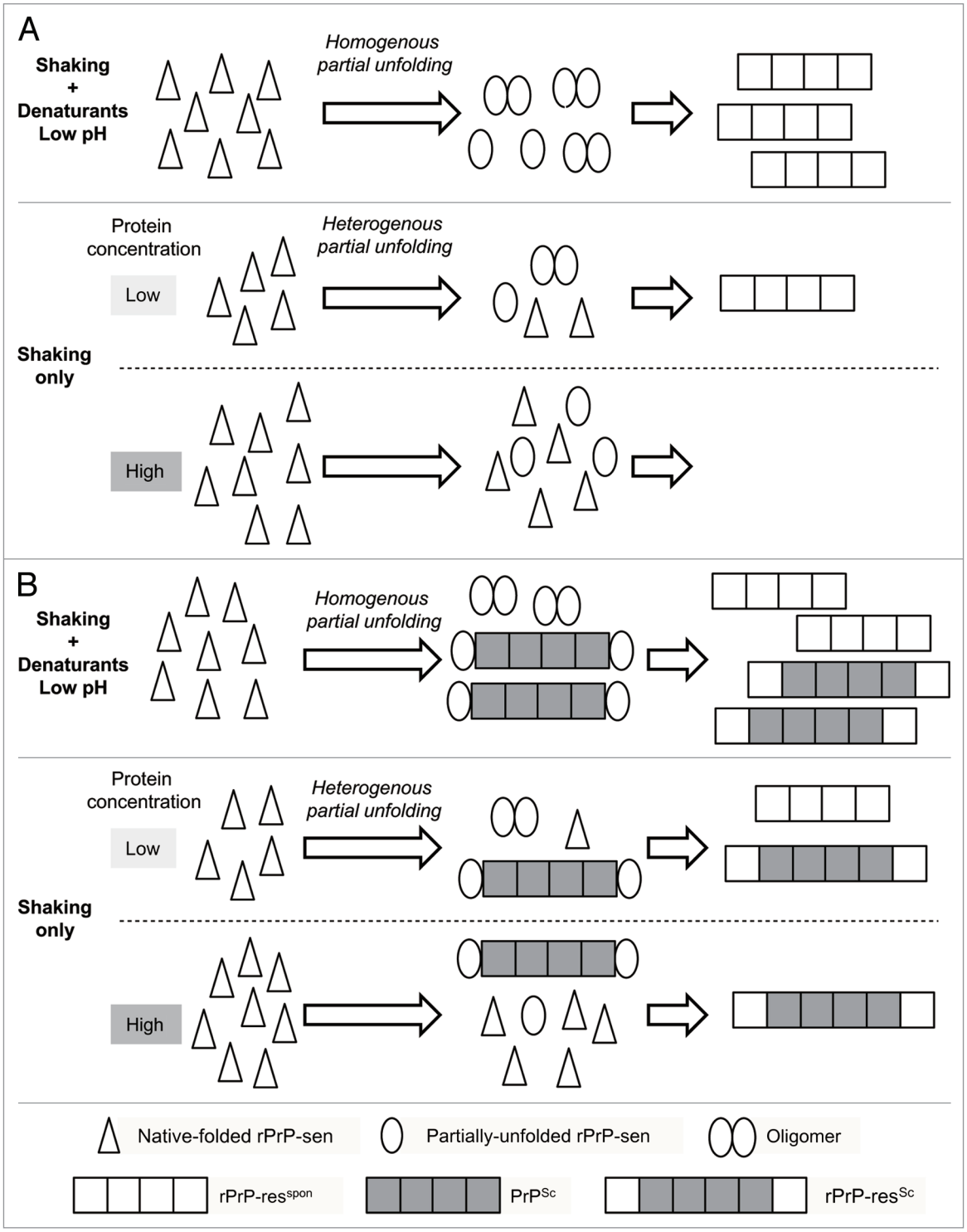

Figure 1. Hypothetical models for the inverse correlation between rPrP-sen concentration and fibril formation in a denaturant-free buffer with shaking in the absence (A) or presence of host-derived PrP^{Sc} (B). Homogeneous partial-unfolding of rPrP-sen is induced in the presence of denaturant or at low pH, leading to an increase of oligomer formation. In contrast, a heterogeneous denaturation status of rPrP-sen is presumed to be inversely proportional to the concentration in a denaturant-free buffer with shaking, resulting in a reduction of oligomer formation. It remains to be determined whether native-folded rPrP-sen can bind to PrP^{Sc} or rPrP-res polymers.

neurological disorders. Moreover, CSF is likely to contain more PrP^{Sc} and fewer impurities than blood. Examining more than 200 CSF specimens from Japanese and Australian patients, we demonstrated that RT-QUIC has greater than 80% sensitivity and absolute specificity for the detection of PrP^{Sc} in the CJD-positive CSF samples.[12] Until now, diagnostic

investigations to evaluate suspected sCJD, although of proven utility, have relied upon non-specific bio-markers, such as the detection of 14-3-3 proteins in the CSF.[25-27] The sensitivity of RT-QUIC was equivalent to and the specificity was much higher than that achieved by 14-3-3 protein measurement. Thus, the RT-QUIC provides a valuable novel means for the antemortem diagnosis of sCJD. Although most of the CSF samples we tested were 129MM, 3/4 129VV and 2/2 129MV CSF samples were positive, suggesting that RT-QUIC using 129M rHuPrP-sen as a substrate is equally valuable in all genetic subtypes of sCJD. Additionally, we recently found that RT-QUIC is potentially useful in the diagnosis of genetic human prion diseases, including Gerstmann-Straussler-Schenker disease (GSS) and fatal familial insomnia (FFI) (manuscript in preparation). While the conversion between PrP-sen and PrP^{Sc} with identical sequences is generally thought to be efficient, our findings suggest that the degree of sequence correspondence between substrate and seed can vary in the RT-QUIC reactions. In support of this concept, we observed that hamster or bovine rPrP-sen can be actively converted into rPrP-res when seeded with sCJD-PrP^{Sc}, albeit with about one log reduction in the detection limit (unpublished data). Furthermore, Orru et al. reported that the use of hamster-sheep chimera rPrP-sen provided for greater sensitivity and less spontaneous fibril formation than was observed with the homologous rHuPrP-sen in the RT-QUIC seeded with vCJD brain homogenate.[28] These results raise the possibility that rPrP-sen may also react to fibrils consisting of other proteins such as beta-amyloid, possibly resulting in a decrease in the specificity of the assay. However, we have yet to experience a single false positive in the RT-QUIC among hundreds of CSF specimens from non-CJD neurodegenerative diseases, including Alzheimer disease, we have tested. Moreover, no increase in ThT fluorescence was observed in the presence of beta-amyloid fibrils artificially formed in vitro (unpublished data). Nevertheless, further studies will be required to completely eliminate the possibility of false positives in the clinical setting. Additionally, the elucidation of the mechanism of rPrP-res^{Sc} formation in the RT-QUIC, including the degree of sequence correspondence, would lead to a better understanding of the molecular basis of prion propagation.

Further Progress in RT-QUIC Technology

Recently, Caughey's group demonstrated that our RT-QUIC could be successfully applied to the detection of hamster and sheep scrapie, deer chronic wasting disease (CWD) and vCJD.[28,29] Additionally, our team has been able to detect BSE at a sensitivity equivalent to that of sCJD (manuscript in preparation). In addition, the RT-QUIC can rapidly determine the relative prion concentration when used in combination with end-point dilution analysis.[29] In another very recent study, Caughey's team showed that enrichment of PrP^{Sc} in plasma by immunoprecipitation employing the PrP aggregate-specific monoclonal IgM antibody 15B3 greatly enhances the sensitivity of RT-QUIC, especially when coupled with a substrate replacement step.[28] Together, these studies demonstrated the wide-ranging application of RT-QUIC to clinical and basic research on human and animal prion diseases.

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