## CHINA CDC WEEKLY





## **PRION DISEASES ISSUE**

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## China CDC Weekly

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This week's issue was organized by Guest Editor Xiaoping Dong.

## Revealing the Mysterious Veil of Prion Diseases Under the Framework of China CDC

Xiaoping Dong<sup>1,2,3,4</sup>

Prion disease (PrD) or transmissible spongiform encephalopathy (TSE) is a group of transmissible and fatal neurodegenerative diseases affecting humans and many animal species. Clinically, PrD, either in humans or in animals, has been documented for more than hundreds of years, e.g., scrapie in the early 18th century and human Creutzfeldt-Jakob disease (CJD) in the early 20th century (1–2). However, the hypothesis and conception of "prions" have been gradually accepted only since the 1980s. As an unconventional infectious agent without nucleic acids, the principle of the "prion" is unsolved conformational changes from host normal membrane protein  $PrP^{c}$  to abnormal pathogenic scrapie-like prion protein ( $PrP^{Sc}$ ) ((3). The hypothesis of the prion concept mostly explains the pathogenesis of PrD; however, there are still many gaps that need to be filled. More importantly, "prion theory" opens a completely new window in biology, possibly highlighting a new type of life.

It has long been known that the tissues of the central nerve system (CNS) from human and animal PrD are infectious; however, animal PrD (e.g., scrapie in sheep and goats) seems to not have the ability to infect humans. The outbreak of bovine spongiform encephalopathy (BSE) in the 1980s and subsequent emergence of variant CJD (vCJD) in the 1990s were one of the largest events in human and animal public health, causing huge panic in society and great economic loss (4). Since then, both the World Health Organization and the World Organization for Animal Health have conducted decades-long surveillance for human and animal PrD. After decades of unremitting efforts, the disease burdens of BSE and vCJD and their threats on public health are efficiently reduced.

As a kind of neurodegenerative disease, PrD shares many similarities as other common neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), but displays remarkable differences in clinical, neuropathology, and laboratory examinations. There are three main types of human PrD according to the etiology, namely the sporadic, genetic, and acquired forms. Lacking typical neurological manifestations, PrD is usually indistinguishable from other neurological diseases, especially in the early stages. The definite diagnosis of human PrD still relies on special examinations of brain tissues, mostly postmortem brains. In the past twenty years, many new diagnostic tools for PrD have been established and comprehensively evaluated, among them, real-time quaking-induced conversion (RT-QuIC) that is able to detect the trace of PrP<sup>Sc</sup> in brain, cerebrospinal fluid, and skin has shown reliable advantages in the diagnosis of PrD (rD (5–6). However, we are still lacking specific prophylactic and therapeutic tools for PrD.

Due to its unique characteristics in biology, prion studies have been one of the research hot topics in biomedicine worldwide. Historically, there were two Nobel laureates in the field of prions and PrD — Prof. C. Gajdusek in 1976 and Prof. S. Prusiner in 1997, who proposed and proved novel biomedical concepts and theories. In the 1990s, prion study received even more attention because of its biological significance and public health importance. The mysterious veil of prions is gradually being lifted.

Talking about prion study in China, we must honorably mention a respective senior scientist, Academician Prof. Tao Hung from China CDC. He performed the first prion experimental rodent assay in the 1980s, at that time human PrD or CJD was poorly understood in the mainland of China. Afterwards, he and the subsequent staff continued and expanded the prion study. A national surveillance for human PrD has been conducted since 2006 under the leadership of China CDC. A series of laboratory tests for PrD diagnosis based on various types of specimens, including RT-QuIC, was developed in the Department of Prion Disease, National Institute for Viral Disease Control and Prevention, China CDC. Those public health practices revealed the features of Chinese PrD patients, meanwhile, supplied irreplaceable laboratory service for hundreds of hospitals in China. Additionally, several basic and applied research studies in the field of prions were conducted, which contributed greatly to understanding the infectivity, pathogenesis, and neuroinflammation of prions.

The papers in this special issue reviewed and summarized the main findings from the prion research group in

China CDC, focusing on the characteristics of Chinese PrD cases based on surveillance, research, and diagnostic platforms for PrD, the disturbance and dysfunction in CNS during prion infection, and the inflammatory reactions in prion infected brains.

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## Characteristics of Different Types of Prion Diseases — China's Surveillance

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## ABSTRACT

This report briefly described the establishment and implementation of national surveillance for human prion disease (PrD) in China. Reported cases came Chinese surveillance network for PrD. from Immunohistochemistry, Western blot, enzyme-linked immunosorbent assay (ELISA), Polymerase Chain Reaction (PCR), and real-time quaking-induced conversion (RT-QuIC) tests were used for the samples of brain, cerebrospinal fluid (CSF), and blood. Diagnosis standard for the PrDs is based on the National Commission of Health (WS/T 562-2017). The study summarized major epidemiological, clinical and laboratory features of more than 2,100 diagnosed different types of Chinese PrD cases. Sporadic Creutzfeldt-Jacob disease (sCJD) is the predominant type of PrD (88.7%). 19 different genotypes of genetic PrDs (gPrDs) were identified, accounting for about 11.3% of all PrDs, revealing ethno-relationships. No iatrogenic CJD (iCJD) and variant CJD (vCJD) was identified. The characteristics of different types of sCJD in China showed similar features as those reported globally, but gPrDs showed an obvious ethnorelationship.

Prion disease (PrD) is a group of fatal and transmissible spongiform encephalopathies (TSEs) affecting humans and species of animals. Human PrDs are classified into sporadic, genetic, and acquired forms. More than 85% of all PrDs were sporadic Creutzfeldt-Jacob disease (sCJD). 10%–15% of PrDs are predominantly inherited involving in different mutations in prion protein (*PRNP*) gene, including genetic CJD (gCJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). Less than 1% of PrDs were acquired, and the majority of patients had definite iatrogenic histories (iatrogenic CJD, iCJD) (1). Since the outbreak of bovine

spongiform encephalopathy (BSE) in cattle in the UK and other countries in the 1980s, a new form of human PrD (variant CJD, vCJD) emerged, caused by consuming food contaminated with the BSE agent.

Human PrD or CJD was rarely recognized and diagnosed in China till the end of the 1980s. Prof. Tao Hung from Chinese Academy of Preventive Medicine, Prof. Shihe Lin from Bethune Medical University, and Prof. Yupu Guo from Peking Union Hospital are the representative pioneers in the field of prion study in China. A collaborating network was set up in the 1990s among several hospitals and research units. After development of the essential laboratory tools for CID diagnosis, a diagnostic center for human PrD was established in 1999 in the Institute of Virology, Chinese Academy of Preventive Medicine. In 2002, the first surveillance program for PrD/CJD was launched in Beijing Municipality, Xi'an City, Guangzhou City, and Changchun City. In 2006, the national surveillance program was officially conducted under the leadership of China CDC, which consisted of the university hospitals and provincial CDCs in 10 provincial-level administrative divisions (PLADs) at the beginning (2-3) and gradually extended to almost all provinces in the mainland of China now. Meanwhile, Chinese national surveillance for PrD/CID joined the international surveillance network under the umbrella of the World Health Organization (WHO), e.g., Surveillance for vCJD in Central and Eastern Europe and China.

## NATIONAL SURVEILLANCE AND DIAGNOSIS NETWORK FOR CJD

Human PrD or CJD was rarely recognized and diagnosed in China till the end of the 1980s. Till now, Chinese surveillance network for PrD consists of 1 national center, 12 provincial units, 15 consultant hospitals (3-4), and gradually extended to almost all provinces in the mainland of China now. The case referring, the data feedback and follow-up survey were conducted according to the surveillance technique

documents. The laboratory tests were performed, including routine neuropathology, immunohistochemistry and Western blot for scrapie-like prion protein (PrPSc) in brains, Western blot for cerebrospinal fluid (CSF) 14-3-3, enzyme-linked immunosorbent assay (ELISA) for CSF tau, prion protein gene (PRNP) PCR and sequencing. Recently, real-time quaking-induced conversion (RT-QuIC) was also applied to the specimens of CSF and skin. The suspected PrD/CJD cases under the national PrD surveillance were diagnosed and subtyped based on the surveillance document issued by China CDC and the diagnostic criteria for CID issued by the National Health Commission. By the end of 2021, 5,078 suspected CID cases were reported to the national center, among them 1,900 were sCJD and 243 were different types of genetic PrDs (gPrDs). No iCJD or vCID cases were identified (Table 1).

## GENERAL FEATURES OF CHINESE **sCJD CASES**

Based on the previously published data (2-6) and the data of the last five years, the onset ages of sCJD patients ranged from 19 to 86 years with the median of 62 years. More cases (roughly 40%) were in the group

of 60-69 years. The gender ratio was 1.06:1 (Males: Females). Except Xizang Autonomous Region (Tibet), sCID cases were identified in all PLADs in the mainland of China. There was no geographic, seasonal or occupational association.

The initial symptoms of sCID cases varied largely. Progressive dementia was most frequently recorded (about 41%), followed by cerebellum and visual disturbances (18%), mental problems (13%), and pyramidal and extrapyramidal symptoms (10%). More neurological abnormalities gradually displayed along with disease progression. Dementia was noted in all sCJD cases. The other four sCJD associated symptoms were also frequently recorded, i.e., visual or cerebellar disturbance (67%), myoclonus (76%), pyramidal or extrapyramidal symptoms (80%), and mutism (39%). The portions of the patients having dementia plus 4, 3, and 2 other neurological symptoms were 19%, 40%, and 41%, respectively.

Periodic sharp wave complexes (PSWC) on EEG were recorded in roughly 50% sCJD patients. Abnormalities on MRI (symmetrical or asymmetrical cortical "ribbon" signs on diffusion weighted imaging DWI, a high signal in the caudate/putamen, or a high signal in the bilateral posterior tuberosity of the thalamus in the proton the density phase) were

TABLE 1. Annual numbers of the referred, sCJD and gPrD cases from 2006 to 2021.

Voar Boforrod			sCID			A normal total or DrD	Annual total PrD	
Tear	Referred	2010	gCJD	FFI	GSS	Annual total grid	Annual total PID	
2006	80	20	1	2	0	3	23	
2007	113	31	3	0	0	3	34	
2008	102	33	1	2	1	4	37	
2009	164	32	3	3	0	6	38	
2010	171	47	5	3	1	9	56	
2011	184	57	5	2	1	8	65	
2012	242	64	8	5	0	13	77	
2013	299	116	9	3	0	12	128	
2014	324	143	8	8	2	18	161	
2015	366	135	17	4	1	22	157	
2016	449	159	16	5	3	24	183	
2017	504	225	20	10	3	33	258	
2018	537	214	16	5	2	23	237	
2019	520	189	25	5	1	31	220	
2020	458	179	18	0	1	19	198	
2021	549	256	12	1	2	15	271	
Total	5,078	1,900	167	58	15	243	2,143	

gPrD

Abbreviation: sCJD=sporadic Creutzfeldt-Jacob disease; gPrD=genetic prion disease; gCJD=genetic CJD; FFI=fatal familial insomnia; GSS=Gerstmann-Sträussler-Scheinker syndrome.

reported in 68% sCJD cases. 77% sCJD cases showed CSF 14-3-3 positive during the clinical course. Increased CSF tau levels (>1,400 pg/mL) were also observed in 87% of the tested cases. Neuropathological and molecular assays of PrP<sup>Sc</sup> in the brain tissues, either postmortem or biopsy, from a small number of sCJD patients showed widely distribution of small granules in brain tissues and type-1 PrP<sup>Sc</sup> molecule.

The majority of sCJD patients progressed rapidly. The clinical durations varied from 2 to 24 months after onset, with the median survival of 5.3 months. Analysis of the durations with other important factors did not show a significant association, including the onset ages, genders, occupations, personal economic situations, clinical symptoms, abnormalities in clinical examinations and laboratory tests.

## GENERAL FEATURES OF CHINESE gPrD CASES

The total Chinese gPrD cases accounted for 11.1% of all diagnosed PrDs, including gCJD, FFI, and GSS (7–8). Genetically, 19 different mutations in *PRNP* were identified in Chinese gPrDs (Figure 1). The most frequent mutation was T188K (9–10), which accounted for 29.8% of all gPrD cases. The mutations with more than 10 cases were D178N (25.7%)

(11–13), E200K (18.8%) (14–15), E196A (7.3%) (16–17) and P102L (6.4%) (18–19). Other mutants with less than 10 cases were E196K (20), V203I (21), R208H (22), V210I, G114V (23–24), R148H, P105L, V180I (25), T183A, and E200G. Four cases were confirmed to contain mutations in the octapeptide repeat (OR) region: one with 7 extra ORs (26), one with 1 extra OR, one with 1 OR deletion (27), and one with 1 octarepeat deletion together with a G114V point mutation in the same *PRNP* allele. Such pattern of mutations in gPrDs was not only completely different from Caucasian cases in European and North American countries, but also different from Japanese and the Republic of Korean cases.

The onset ages of Chinese patients with gPrDs were generally younger than that of sCJD patients, with the median of 50 years old (19,85). Different gPrDs showed obvious diversity in their onset age. The median onset ages of P102L GSS (50 years) and D178N (51 years) FFI were younger than those of T188K (61 years), E196A (61 years) and E200K (57 years) gCJD. 78% of P102L GSS and 54% of D178N FFI cases had a definite family history, whereas only approximately 15% of T188K and E200K gCJD patients recorded family history. Unlike sCJD, some types of gPrDs showed geographic association, e.g., more D178N FFI cases in Henan and Guangdong, while more cases of E200K cases in the northern



FIGURE 1. Schematic structure of PrP protein and 19 different genotypes of Chinese gPrDs.

Note: The signal peptide at N-terminus, GPI anchor at C-terminus, five OR, three  $\alpha$ -helix regions, two  $\beta$ -sheet regions are shown inside. The serial numbers of amino acid are indicated above the schematic structure. The top five frequently observed gPrDs are illustrated in the upper part, with their proportion (%), the median of onset age (year, y), the median of duration (month, m). The rest of mutants are shown in the lower part.

Abbreviation: OR=octapeptide repeat; gCJD=genetic Creutzfeldt-Jacob disease; PrP=prion protein; gPrDs=genetic prion diseases; GPI=glycosylphosphatidylinositol; GSS=Gerstmann-Sträussler-Scheinker syndrome.

provinces.

Besides the great differences in clinical manifestations between gCJD, GSS, and FFI, the profiles of EEG, MRI, CSF 14-3-3, and CSF tau among the gPrD patients with different mutations were also different. PSWC on EEG was identified in 48% E200K gCJD cases, but low in E196A (25%), T188K (27%), P102L (20%), and extremely rare in D178N (2%). Special abnormalities on MRI were frequently recorded in most types of gPrDs, i.e., P102L (92%), T188K (78%), E196A (73%), E200K (85%), but infrequently in D178N (25%). Positive CSF 14-3-3 was detected more often in gCJD patients (62% in T188K, 75% in E196A, 71% in E200K), but less in D178L FFI (39%) and P102L GSS (41%) cases. Increased CSF tau was observed in a small portion of P102L (24%), but frequently in D178N (59%), T188K (54%), E196A (80%), and E200K (68%).

The survival time of P102L cases was notably longer than that of the others, with a median (50% percentile) of 16 months. 60% of P102L cases survived longer than 12 months and 40% longer than 24 months. The median survival time of D178N cases was 11 months, among them 30% cases survived longer than 1 y, whereas those of T188K (4 months), E196A (6.5 months), and E200K (6 months) were clearly shorter. The ratios of the cases alive longer than 1 y after onset were 6.5% in T188K, 17% in E196A, 20% in E200K, respectively. Taken together, the gPrD cases showed an obvious difference in the characteristics of epidemiology, clinic and laboratory compared to sCID cases.

## THE PROFILES OF POLYMORPHISM OF CODON 129 AND CODON 219 AMONG THE PATIENTS WITH DIFFERENT DISEASES

Two polymorphisms in *PRNP*, codon 129 and codon 219, affect the susceptibility and phenotype of

PrDs. Previous studies have confirmed that East Asians have predominant genotype of M129M (92%–95%) compared to Caucasians (50%–70%). *PRNP* sequencing of more than 5,000 referred cases in the national surveillance for PrD also proposed absolutely predominant patterns of M129M and homozygote of glutamic acid at codon 219 (E219E). Compared to the group of non-CJD, both sCJD and gPrD cases showed even higher ratios of M129M and E219E (Table 2).

## DISCUSSION

One of the most significant achievements of Chinese national PrD surveillance is comprehensive description of the epidemiological, clinical and laboratory features of Chinese PrDs. It enriches the overall understanding of human PrDs. sCJD is the predominant type of PrDs in China, showing similar characteristics to those of other countries. About 10% PrDs cases are gPrDs consisting of 19 different genotypes. Notably, the profile of Chinese gPrDs is identical not only to that of Western countries but also to our neighboring ones, i.e., Japan and the Republic of Korea. T188K and E196A gCJDs are popular in China, but extremely rare in other countries. On the other side, some frequently reported gPrDs in Japanese, such as P105L GSS and M232V gCJD, are rare in Chinese. It verifies again the ethno-correlation of distributions of PRNP variants. There is no vCJD case in the mainland of China, which may, from another angle, reflect a BSE-free environment.

Our surveillance for human PrDs promotes the recognition and diagnosis of such rare diseases. Along with the implementation of PrDs surveillance, more and more hospitals from various provinces in the mainland of China referred CJD cases and the annual case numbers referred to the surveillance center were over five hundred in the past five years. Our PrDs surveillance also proposes strong technique support for diagnosis and differential diagnosis of PrDs for

TABLE 2. Proportions of the polymorphism at codor	129 and 219 in PRNP in different disea	ses from 2006 to 2021 (%)
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Disease –	-	Codon 129		Codon 219		
	M129M	M129V	V129V	E219E	E219K	K219K
sCJD	98.5	1.5	0	98.9	1.1	0
gPrD	98.2	1.8	0	98.7	1.3	0
non-CJD	96.8	3.2	0	93.5	6.5	0
Referred	97.6	2.4	0	96.3	3.7	0

Abbreviation: PRNP=prion protein gene; sCJD=sporadic Creutzfeldt-Jacob disease; gPrD=genetic prion disease.

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hundreds of hospitals over China. Thousands of laboratory tests of clinical samples each year, countless communications and consultants with physicians, timely feedback of the diagnosis and disposal suggestion for each referred case under the national PrDs surveillance set up a good example for the combination of clinical and preventive medicine. Our surveillance program also functions as a platform to communicate with the family members and relatives of PrD patients, providing medical consultant and daily care suggestions and greatly alleviating the fear of this unfamiliar disease due to its infectious potential.

As a neurodegenerative disease, there is still a lack of specific therapeutic or prophylactic tools for any type of PrD. The wide distribution of infectious prions in the central nerve system, eyeballs, and some other peripheral lymph tissues has caused great concern about iatrogenic infection, e.g., neurosurgical operation, organ transplantation, and even blood transfusion. Moreover, the impacts and threats of BSE and other animal prion diseases, such as chronic wasting disease in Cervidae, on public health of humans are still long-standing. Long-term surveillance for human and animal PrDs is still needed.

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# Establishment of a Special Platform for the Research of Prion and the Diagnosis of Human Prion Disease — China's Studies

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## ABSTRACT

The studies of prions and prion disease usually need many special platforms and techniques that differ from those for classical microbes. Search of new biomarkers and establishment of new methods for the diagnosis of human prion diseases are priorities in the field of prion study.

In this report, the main platforms in Chinese Center for Diseases Control and Prevention for prion study and diagnosis were introduced. Some findings of crossspecies transmission and some potential anti-prion candidates were also discussed. Several prion-infected rodent models based on wild-type and transgenic animals were developed. Two in vitro protein amplification methodologies, protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC), were established and applied in many different prion studies, whilst RT-QuIC with cerebrospinal fluid and skin specimens was applied in the diagnosis of human prion diseases. PMCA can help prion strains overcome species barrier, efficiently propagating in vitro and inducing interspecies infection in vivo. Some natural components, such as resveratrol and 3,4-dihydroxybenzalacetone (DBL), showed antiprion activities both in vitro and in vivo.

Establishment of many special platforms efficiently deepens the prion studies and provides new sensitive tools for the surveillance and diagnosis of human prion disease.

Prion infection shares some similarities with the infections of other microorganisms (e.g., viruses and bacteria), such as fulfilling Koch postulates, having strain features, showing species barriers, etc. On the other hand, prion infection has unique characteristics, such as not eliciting host specific humoral and cellular immunoresponses without pathogenic-associated nucleic acids, etc. (1).

Prion disease is also a zoonotic disease. Transmission across different species of animals may occur naturally and artificially. The most famous example is the outbreak of bovine spongiform encephalopathy (BSE) in cattle in the United Kingdom (UK) and other European countries in the 1980s' caused by feed consisting of meat and bone meal (MBM) being contaminated with scrapie agents. Subsequently, a new type of human prion disease, variant Creutzfeldt-Jacob disease (vCJD), emerged in the UK and many other countries worldwide due to consuming BSEcontaminated beef (2).

Because of the uniqueness of prion biology and special physiopathology of prion disease, prion study and disease diagnosis usually need many special platforms and techniques that are different from classical microbes. Transgenic techniques supplied the basis for the development of prion theory and protein amplification techniques *in vitro* which accelerated the speed of prion research. In this report, the main platforms in Chinese Center for Diseases Control and Prevention for prion study and transmissibility of different prion strains in bioassays were summarized (Figure 1).

#### **PRION INFECTED RODENT MODELS**

Small experimental rodents, e.g., mice and hamsters, are commonly used for some strains of prions. The first prion mouse model in China was imported and established by Prof. Tao Hung in the Institute of Virology, Chinese Academy of Preventive Medicine in the 1980s.

Afterwards, several scrapie infected rodent models established have been and the clinical, neuropathological, pathogenic, and infectious characteristics have been comprehensively analyzed, including a hamster infected with scrapie hamsteradapted strain 263K, a C57 mouse infected with scrapie mouse-adapted strain 139A, and a C57 mouse infected with scrapie mouse-adapted strain ME7.

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FIGURE 1. The timeline of the key milestones of prion research and surveillance in China. Abbreviation: CSF=cerebral spinal fluid; CJD=Creutzfeldt-Jacob disease; PMCA=protein misfolding cyclic amplification; RT-QuIC=real-time quaking-induced conversion.

Despite different median infective dose and incubation times, the major neuropathological and pathogenic features of different inoculating ways were the same. Later, other scrapie mouse models were established, such as the C57 mouse infected with agent 22L, C57, Balb/c, and CD1 mouse infected with the lysate of prion infected SMB-S15 cells, and C57 mouse infected with PMCA product (*3*).

We have also imported or developed several transgenic (Tg) mouse strains, such as Tg mouse knockout prion protein gene (*PRNP*), Tg mouse with human *PRNP*, Tg mouse with hamster *PRNP*, etc. Recently, based on the Tg mouse of knockout *PRNP*, we established two strains of Tg mice expressing chimeric MiniSOG-MusPrP aiming to study the morphology of scrapie fibril agent (SFA) and a strain of Tg mouse expressing human *PRNP* with T188K mutant (4). The relevant studies are ongoing.

#### **PMCA AND APPLICATION**

PMCA is a technique by mixing scrapie-like prion protein ( $PrP^{Sc}$ ) in the tissues of infected animals with cellular prion protein ( $PrP^{c}$ ) from normal brains under the condition of cyclic processes of alternative sonication and incubation in a special facility, a large amount of normal  $PrP^{c}$  can be converted into  $PrP^{Sc}$  in two to four days. Two PMCA methods were developed in our laboratory, direct PMCA aiming to detect  $PrP^{Sc}$ in the tissue samples and serial PMCA aiming to continual passage of  $PrP^{Sc}$  *in vitro*. Compared with the routinely used Western blot, the detection sensitivity of PMCA for  $PrP^{Sc}$  in the brains of prion infected experimental rodents increased  $10^2-10^3$  folds (5).

PrP<sup>Sc</sup> propagation was elicited in muscle and spleen tissues, which were approximately 10<sup>-2</sup> lower than brains. Traces of PrP<sup>Sc</sup> replication were also detected in the intestine and kidney tissues of infected hamsters, which were about  $10^{-4}$  lower than brains.

Pyridine nucleotides, a group of coenzymes ubiquitous in biosynthesis and metabolism, are associated with the aggregation of recombinant prion protein (rPrP). By using PMCA, we confirmed that PrP<sup>Sc</sup> from the scrapie infected rodent brains propagated much more efficiently in the presence of reduced pyridine nucleotides, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and nicotinamide adenine dinucleotide hvdrogen (NADH), but remained unchanged in the presence of oxidized form of pyridine nucleotides, nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), and vitamin C. The enhancement of reduced pyridine nucleotides on PrPSc replication was also observed in the PMCA using recombinant hamster prion protein (PrP) as substrate. It supplies the molecular basis for the involvement of reduced pyridine nucleotides in prion replication (6).

#### **RT-QuIC AND APPLICATION**

Real-time quaking-induced conversion (RT-QuIC), has been developed and greatly improved the detection limit of PrPSc (7). Cerebral spinal fluid (CSF) RT-QuIC has been also set up in Chinese national surveillance (8-9). The 1st generation of RT-QuIC the full-length recombinant hamster PrP used (rHaPrP23-231) as the substrate and the 2nd generation used the truncated hamster PrP (rHaPrP90-231). Validation with clinical CSF samples of definite sporadic CJD (sCJD) patients with neuropathological diagnosis, the established RT-QuIC (the 2nd generation) showed good sensitivity (96.67%) and specificity (100%). Wide application of CSF RT-QuIC in CJD surveillance also revealed high positive rates in probable sCJD cases, making it acceptable in the national diagnostic criteria for sCJD (9). Two separate studies illustrated that the positive rates (30%–32%) of CSF RT-QuIC in genetic prion disease were generally lower than those of sCJD. Based on the data of five commonly identified genetic prion diseases in China, the positive rates from highest to lowest were P102L Gerstmann-Sträussler-Scheinker syndrome (GSS) (60%–61.5%), E200K genetic CJD (gCJD) (40%–44%), E196A gCJD (37.5%–40%), T188K gCJD (25.7%), and D178N fatal familial insomnia (FFI) (15.8%–16.2%) (10).

Recently, after collaborations with Prof. Zou from Case Western Reserve University, traces of prions in skin specimens of sCJD patients and scrapie infected rodents were verified to be sensitively detectable by RT-QuIC (11-12). Validation of skin RT-QuIC with dozens of skin specimens from sCJD cases and non-CJD patients showed 100% specificity and 95.5% sensitivity (13). Compared to brain biopsy and lumbar puncture, skin biopsy is much less invasive.

One of the neuropathological characteristics of FFI cases is the significantly lower amount of  $PrP^{Sc}$  in the brain tissues (14–15). Positive results of RT-QuIC were detected in the postmortem brain tissues of FFI cases at 10<sup>-5</sup> dilution. The RT-QuIC reactivities (e.g., lag time and fluorescent peak) of FFI brains were significantly weaker than that of sCJD (16). High sensitivity and wide tissue adaption of RT-QuIC make it become a powerful diagnostic tool for prion disease and prion study.

## IDENTIFICATION AND VALIDATION OF THE DIAGNOSTIC BIOMARKERS IN CSF

Our proteomics assay of the CSF samples of sCJD revealed a significant increase of calmodulin (17). A later study confirmed that the expressions of calmodulin in the brains of scrapie infected rodents were also upregulated, highlighting the possibility of setting up a new tool for diagnosis of sCJD (18). Western blot positively identified CSF calmodulin in 70% (28/40) of probable sCJD and 73.3% (22/30) of pathologically definite sCJD, whilst in 22.5% (9/40) of non-CJD and 20% (6/30) of pathologically excluded non-CJD. Logistic regression established a significant correlation between the CSF calmodulin signal and total CSF tau level (19).

Six tau isoforms with different molecular weights have been found in the brains, differing by the number

of exon-2 (29-aa) and/or exon-3 (29-aa) insertion in the N-terminus and exon-10 (31-aa) in the C-terminal half of the protein. We have separately prepared the specific polyclonal and monoclonal antibodies against exon-2, -3 and -10 of tau (20). A 65 kDa-large band was detected in the CSF samples of sCJD patients, in the reactions of the antibodies to exon-2 and exon-10, revealing good correlation between positive CSF 14-3-3 and typical abnormality in electroencephalogram (EEG). Majority of the increased tau in the CSF of sCJD cases was derived from the tau isoforms with exon-2 and exon-10 segments (21).

## **CROSS-SPECIES TRANSMISSION**

Species barriers of prion infection in experimental mice and hamsters show an interesting phenomenon. Intracerebral infections of mouse-adapted scrapie strains 139A and ME7 onto hamsters could cause typical prion disease after long incubation periods. Contrary to the short lag time of homologous infection of hamster adapted scrapie strain 263K (66-80 days), the hamsters with heterologous infection by strains of 139A and ME7 displayed typical diseases after 358-450 and 460-530 days, respectively. The molecular characteristics of the newly formed PrP<sup>Sc</sup> in 139A and ME7 infected hamsters were obviously distinct from their original mouse ones, whilst greatly similar to that of hamster strain 263K. On the other hand, inoculation of the hamster-adapted scrapie strain to mice was unable to induce prion disease. Crossspecies transmission of prions between mouse and hamster under the experimental condition was a oneway direction, transmissible from mouse to hamster but not transmissible from hamster to mouse (22).

PMCA verified that both mouse-adapted strain 139A and hamster-adapted strain 263K could use brain homogenates of opposite species to form PrP<sup>Sc</sup>. These two newly formed interspecies PMCA prions could stably propagate in the subsequent serial PMCA passages; meanwhile, they lost their original molecular characteristics but possessed the new host features. Inoculations of new PMCA prions to the heterologous animals efficiently caused the typical prion diseases. Unlike the prolonged lag time of interspecies infection of mouse strain 139A on hamsters, the incubation time hamsters infected with 139A-induced the of interspecies PMCA prion was much shorter. This suggests that PMCA can help prion strains overcome species barrier, efficiently propagating in vitro and inducing interspecies infection in vivo (Figure 2) (23).



FIGURE 2. Schema of interspecies transmissions of mouse- or hamster-adapted scrapie prions onto opposite animals directly or via PMCA. Scrapie agents in the brain tissues of infected animals (brain-prion) or via PMCA amplification (PMCA-prion) were separately inoculated into respective animals intracerebrally. Abbreviation: PMCA=protein misfolding cyclic amplification.

## ANTI-CYTOTOXICITY AND ANTI-INFECTIVITY OF PRIONS

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a natural polyphenolic phytoalexin from many fruits and vegetables, showing a number of health benefits, such as neuroprotection, cardioprotection, hepatoprotection, anti-inflammation, and cancer chemoprevention. Exposure of prion infected cell line SMB-S15 to resveratrol remarkably reduced and even removed cellular PrP<sup>Sc</sup> in a dose-dependent manner. Prion replication in SMB-S15 cells treated with 5 and 10 µmol/L resveratrol was irreversible after the drug withdrawal. Inoculation of the lysates of resveratrol-treated SMB-S15 cells on mice completely lost the infectivity, proposing a valuable therapeutic potential (24).

Two other stilbene compounds, pterostilbene (Pte) and piceatannol (Pic), also revealed anti-prion activities *in vitro* study. In the level of cultured cells, obvious suppressions on PrP<sup>Sc</sup> replication in SMB-S15 cells were observed, in which resveratrol (Res) was the most active one, followed by Pic and Pte. The inhibitive activities of those three stilbenes on the brain-derived prion from agent 263K-infected hamster were also identified in hamster PrP-based PMCA and RT-QuIC. Molecular binding of stilbene compounds with mouse PrP was proven by Biacore assays, highlighting an association between clearance of prions and molecular binding (25).

Anti-prion activity was also observed in other natural compounds, such as 3,4-dihydroxybenzalacetone (DBL), a small catechol-containing compound purified from the ethanol extract of Inonotus obliquus. After exposure to 10 µmol/L of DBL, the level of PrP<sup>Sc</sup> in SMB-S15 cells was significantly decreased. The levels of reactive oxygen species and hydrogen peroxide were decreased, whereas the levels of some antioxidant factors, such as heme oxygenase 1, glutamate-cysteine ligase catalytic, and glutamate-cysteine ligase modifier, were significantly increased. The activities of total glutathione and superoxide dismutase, and the levels of unfolded protein response-related proteins were upregulated. Such anti-cytotoxicity phenomena were only detected in DBL-treated prion infected cells but not in their normal partner cells. Another compound, vanillic acid, also displayed similar anti-prion activity in vitro (Figure 3).

#### DISCUSSION

Because of the unique biological features, study and diagnosis of prions and prion disease are completely different from other microorganisms and infectious



FIGURE 3. Scheme of the activities of anti-prion replication and anti-prion cytotoxicity of resveratrol, 3,4-DBL, and vanillic acid inhibit the replication and infectivity of prions via direct molecular interaction while alleviating the prion cytotoxicity by mediating various cellular agents and pathways.

Abbreviation: PrP<sup>sc</sup>=scrapie-like prion protein; PrP<sup>c</sup>=cellular prion protein; ROS=reactive oxygen species; SOD=superoxide dismutase; ER=endoplasmic reticulum;DBL=dihydroxybenzalacetone.

diseases. Up to now, animal assays are still one of the most important platforms for prion study. Numerous types of transgenic mice including *PRNP* and other associated genes greatly help to understand the pathogenesis. PMCA techniques markedly increases the speed of prion replication, and PMCA-generated prions are usually infectious, showing a great advantage for evaluating prion infection. RT-QuIC usually reflects the fiberizing ability of prion, and its product usually does not have infectivity. Thus, animal bioassays are still an indispensable tool for prion research.

Neuropathological and PrP<sup>Sc</sup> tests based on postmortem brains are the pathway for definite diagnosis of human prion diseases. However, brain tissues obtained from biopsy or autopsy have been limited since the establishment of the surveillance network. In addition to the traditional culture of the Chinese people, fear of prion infectivity is another reason for refusing to perform biopsies or autopsies in hospitals. In the future, publishing guidelines for biopsies or autopsies of sCJD patients will be helpful to obtain brain tissues. In the meantime, verifying the accuracy and reliability of skin RT-QuIC assay and applying skin RT-QuIC assay to partially replace brain tissue pathology is also an ideal option in China.

For decades, screening of specific biomarkers in CSF and other peripheral specimens has been continually conducted. Only two CSF proteins, 14-3-3 and tau, are included in the diagnostic criteria. However, the diagnostic sensitivity and specificity of those two proteins in CSF vary largely among the different laboratories. Further searching of the diagnostic biomarkers in other easily obtained samples. i.e., blood and urine, with novel techniques is valuable. The diagnostic significance of CSF RT-QuIC for sCJD has been documented. More recently developed skin RT-QuIC reveals even better diagnostic sensitivity and specificity for sCJD. Further standardization and industrialization of RT-QuIC will improve its usage clinically.

The outbreak of BSE and emergence of vCJD alarm the possibility of the transmission of prion strains from other species of animals to humans. Chronic wasting disease is considered as also having potential. The mechanism of cross-species transmission of prions is one of the hot topics in the field of prion study. Studies based on PMCA have verified that PMCA can help prion strains to transmit interspecies and even generate new prion strains *de novo*, highlighting that prions affecting new susceptible species may be generated by artificial extreme environments, such as the production process of meat and bone meal. PMCA, RT-QuIC, and other new methodologies supply useful tools for fast screening of potential materials for research and development of therapeutic and prophylactic drugs. However, to date, no specific, effective prophylactic regimens or therapeutic tools have been established for human or animal prion diseases, which still needs more effort.

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## Activation of Innate Immunity and Autophagy in Brain Tissues with Prion Disease and Degradation of Abnormal PrPs in Cells — China's Studies

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## ABSTRACT

Unlike infectious diseases caused by conventional microbes, there are no detectable specific humoral or cellular immunoresponses to prion infection. However, extensive and active gliosis is observable in affected brain regions along with significant deposits of scrapielike prion protein (PrPSc). Here, we summarize our studies of vibrant activation of host non-specific immunocomponents and autophagy in the microenvironment of prion infected brains. Activation of the brain's innate immunity and autophagy upon prion infection reflect non-specific host defense systems attempt to dispose of accumulated prions. Vibrant elevation of neuroinflammation leads to neuron injury.

The neuropathology of prion disease (PrD) has two completely different scenarios - one is severe depression and death due to severe neuron loss, cell apoptosis, and extensive spongiform degeneration, and the other is activation, expressed as gliosis, activated autophagy, and non-specific immunity. These polarized neuropathological scenarios are continually observable, even at the terminal stage of most types of human and animal PrDs. Prion infection does not elicit any detectable specific humoral or cellular immunoresponse, but host innate immunity appears to be persistently activated in infected brains, possibly for clearance of prions. Accumulated scrapie-like prion protein (PrPSc) may further stimulate strong nonspecific immunoresponse. However, overreactive brain immunoresponse may eventually deteriorate pathological processes of PrD. In this report, we summarize major findings of our studies on overreaction of innate immunity and inflammation.

## MICROGLIOSIS AND ASTROGLIOSIS

Microglia are resident mononuclear phagocytes of the central nerve system (CNS), comprising approximately 10% of CNS cells. Based on scrapieinfected rodent models, substantial microgliosis is noticeable in infected brains, regardless of amounts (marked by Iba1) or activities (marked by CD68), and shows а time-dependent increase. Histologic distribution of activated microglia co-localize with PrP<sup>Sc</sup> in various brain regions (1). Abnormal activation of microglia can also be detected in the cortex, thalamus, and cingulate gyrus regions of sporadic Creutzfeldt-Jakob disease (sCJD) and G114V genetic CJD (gCJD) patients with large amounts of PrPSc deposits, but are not detected in the brains of D178N fatal familial insomnia (FFI) patients with fewer PrPSc deposits (2-4). Brain CXC3L1 is a negative regulator for activation of microglia that is downregulated in prion infected rodent models, highlighting a possible regulating pathway of fractalkine signaling deficiency (1).

Exposure of supernatant from lipopolysaccharide (LPS)-treated microglia BV2 cells and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) onto scrapie infected cell line SMB-S15 induces lower cell viability than its normal partner cell line SMB-PS. An activated phosphorylated form of mixed lineage kinase domain-like protein (p-MLKL, a marker of necroptosis) was observed both in SMB-S15 cells and in cortical tissues of patients with sCJD and gCJD. The decreased cell viability of SMB-S15 and the increased p-MLKL induced by TNF- $\alpha$  can be completely rescued by a necroptosis specific inhibitor necrostatin-1. Removal of PrPSc propagation in SMB-S15 cells by resveratrol partially rescues cellular tolerance to TNF-a. Overreactive microglia may have more influence on cells containing PrPSc via activation of the necroptosis pathway (5).

Glial fibrillary acidic protein (GFAP) distributes

exclusively in cytoplasm of mature astrocytes. One of the functions of GFAP is in the interaction of astrocytes with other cells that are required for the formation and maintenance of the insulating layer (myelin). Specific molecular interaction was found between prion protein (PrP) and GFAP, for both recombinant and native PrP (including PrP<sup>Sc</sup>) (6). Overreactive proliferation of astrocytes during prion infection can lead to overexpression of a small heat shock protein,  $\alpha$  B-crystallin (7).

## ABERRANT ACTIVATION OF COMPLEMENT SYSTEM

Abnormally enhanced complement systems have been repeatedly identified in prion infected brains, including total complement activity levels and major components (C1q and C3), highlighting activation of complement classical pathway the (Figure 1). Membrane-attacking complexes (MAC) in infected show remarkable time-dependent brains also deposition during the incubation period, indicating activated terminal persistently complement components. MAC-specific signals overlap with neurons, while C3 distributed in astrocytes and microglia possibly associate with activation of those cells. (8). The alternative pathway (AP) for activation of the complement system in the CNS is also activated

during prion infection. Key triggering elements and positive regulation of AP, complement factor B (CFB), and complement factor P (CFP) increase significantly in brain tissues of scrapie infected mice in a time dependent manner (9).

Unlike in brain tissues, complement components in cerebrospinal fluid (CSF) from sCJD patients are reduced or unchanged compared to non-CJD cases. Proteomic assays found that two components (C9 and CFB) are significantly decreased and three (C4b, C7, and C2) are non-significantly decreased in CSF samples of sCJD patients (10). Complement hemolytic activity (CH50) has significantly lower activity in CSF samples in various types of human prion diseases. Complement homeostasis happens differently in brains and in CSF of prion disease patients. Contrary to the wide range of CH50 values among sCJD patients, the CH50 values in genetic prion diseases, including T188K gCID, E200K gCID, and D178N FFI, are much narrower, which reflects distinct pathogeneses of sporadic and inherited prion diseases (11).

## ABNORMAL ALTERATIONS OF CHEMOKINES AND CYTOKINES

Along with activation and proliferation of microglia and astrocytes during prion infection, levels of many chemokines and cytokines in infected brains are also



FIGURE 1. Schema of activation and level changes of components of innate immunity and neuroinflammation in the central nervous system during prion infection.

Note: PrP<sup>Sc</sup> amyloid stimulates the activation of astrocytes and microglia, and secretes various complement components, chemokines, cytokines, and inflammatory factors. Complement components can form membrane-attacking complexes (MAC) on the surface of PrP<sup>Sc</sup> infected neurons through classical and alternative pathways, subsequently inducing neuronal damage. Various chemokines, cytokines, and inflammatory factors can bind to receptors on the surface of neuronal membranes to activate a series of signaling pathways in neurons that induce neuronal death when infected with PrP<sup>Sc</sup>. Abbreviation: PrP<sup>Sc</sup>=scrapie-like prion protein; MAC=membrane-attacking complexes; CFB=complement factor B; CFP=complement factor P.

increased — for example, interleukin (IL)-1  $\beta$ , IL-6, and TNF- $\alpha$  accompany marked PrP<sup>Sc</sup> deposits (Figure 1) (*1*–2). Many chemokines in supernatant from cultured microglia increased significantly when stimulated with LPS, strongly implying a critical role for microglia activation in upregulated chemokines (5).

Transcriptional and translational levels of interferon gamma-induced protein 10 (IP10) and macrophage colony stimulating factor (M-CSF) were repeatedly upregulated in prion infected brains and prion infected cell lines. Slight but significant increases of IP10 levels were identified in the CSF samples of sCJD patients compared to non-CJD patients (12). Receptors CXCR3 for IP10 and CSF1R for M-CSF were increased in prion infected brains. Upregulated ligand signals were detected in neurons and microglia, while increased receptors are primarily distributed in neurons, which indicates that microglial cells are the main secretory cells for IP10 and M-CSF, with neurons as the major target cells. Neuropathologically, IP10 and CXCR3, as well as M-CSF and CSF1R, accumulate in brain regions with more PrPSc deposits. Molecular binding activities of PrP with IP10/CXCR3 and M-CSF/CSF1R were also addressed. Clearance of PrPSc replication in prion infected cells partially converts overexpression of IP10 and M-CSF.

## **ACTIVATION OF AUTOPHAGE**

There are two main mechanisms for quality control of protein expression in eukaryotic cells: ubiquitinproteasome and autophagy-lysosome pathways. Enhanced macroautophagic system can be clearly identified in the brains of prion disease patients and in prion infected cell lines or cells expressing PrP mutants. Deep repression of the mammalian target of the rapamycin (mTOR) pathway - an essential negative regulatory mechanism — has been shown to be closely associated with autophagy activation. Blockage of macroautophagic activity by a specific inhibitor (bafilomycin A) efficiently slows degradation of abnormal PrPs and PrP<sup>Sc</sup> in cultured cells. Activation of the autophagic system begins at early stages of prion infection, likely attempting to resolve abnormal PrP aggregation. Neuron damage will occur once PrP<sup>Sc</sup> replication and deposits exceed the capacity of the host's clearance system, including by autophagy (13).

Knockdown of ATG5 and treatment of three autophagic inhibitors induces a significant increase of PrP<sup>Sc</sup> in prion infected cell lines and an increase of

mTOR levels. F-box and WD repeat domains containing 7 (FBXW7) constitute one of the four subunits of the E3 ubiquitin protein ligase and targets mTOR for ubiquitination and degradation. Brain FBXW7 levels in scrapie infected animals and prion infected cells were upregulated at early stage. Knockdown of cellular FBXW7 remarkably inhibited autophagic flux and increased PrP<sup>Sc</sup> accumulation. Enhanced expression of FBXW7 and subsequent activation of autophagy via downregulation of mTOR at early stage acts to clear invasive prions (*14*).

AMP-activated protein kinase (AMPK) is a serine/threonine kinase functioning as a positive regulator for autophagy by phosphorylating its downstream Unc-51-like autophagy activating kinase 1 (ULK1) at specific sites. Increases of brain AMPK and ULK1, as well as their phosphorylated forms AMPK-Thr172 and ULK1-Ser555, occurred at early stages of scrapie infected hamsters. Liver kinase B1 (LKB1), which mediates AMPK activation, is also increased in the infected brains at early and middle disease stage. Upregulation of activators in brains correlate with reduction of mTOR and activation of autophytic activity during prion infection. Upregulation of AMPK and ULK1 were seen in prion infected cell lines and knockdowns of cellular ULK1 reduced autophytic activity. The enhanced brain AMPK-ULK1 pathway reflects an active host response to prion infection (15).

Mitophagy is a special, selective autophagy process that maintains mitochondrial health and eliminates damaged mitochondria. Marked increases of Pink1 and Parkin were observed in prion infected cell lines. Activated Pink1/Parkin pathway modifies outer membrane proteins on damaged mitochondria via phospho-ubiquitin polyubiquitin chains, which reflects activated autophytic flux. Inhibition on the expressions of either Pink1 or Parkin in prion-infected cells can relieve autophagic flux. Aberrantly enhanced Pink1 and Parkin were also observable in different brain regions of scrapie-infected mice. Pink1- and Parkinpositive cells distributed more in the areas with amounts of PrP<sup>Sc</sup> in scrapie infected mice, indicating an association between PrPSc deposits and activation of mitophagy (Figure 2) (16).

## ENHANCEMENT OF OTHER ELEMENTS ASSOCIATED NEUROINFLAMMATION

 $\alpha$  1-antichymotrypsin ( $\alpha$  1-ACT) is an acute-phase inflammatory protein.  $\alpha$  1-ACT levels are significantly



FIGURE 2. Schema of chaperone-mediated autophagy, macroautophagy, and mitophagy in cytoplasm to degrade PrP mutants and PrP<sup>sc</sup>.

Note: Abnormal changes in PLK family components can enhance the expression of cellular LaAMP2a and Hsc70, which can form molecular complexes with intracellular PrP mutants and induce autophagy. PrP<sup>sc</sup> activated FBXW7 and AMPK signaling pathways, thus induce macroautophagy. PrP<sup>sc</sup> can also activate Pink1-Parkin signaling pathways and induce mitochondrial dysfunction and mitophagy.

Abbreviation: PrP=prion protein; PrP<sup>sc</sup>=scrapie-like prion protein; PLK=polo-like kinase; LaAMP2a=lysosome-associated membrane protein type 2A; Hsc70=heat shock cognate protein 70; FBXW7=F-box and WD repeat domains containing 7; AMPK=AMP-activated protein kinase.

increased in brain tissues of scrapie-infected rodents in a time dependent manner. Increased  $\alpha$  1-ACT shows morphological co-localization with PrP<sup>Sc</sup> deposits in infected brains and is identifiable in astrocytes, microglia, and neurons (17). Galectin-1 (Gal-1) is implicated in the regulation of innate and adaptive immunity. Remarkable increases in brain Gal-1 is observed in many scrapie-infected rodents at terminal disease stages. In postmortem brains of human prion diseases, Gal-1 levels were upregulated. More *S*nitrosylated forms of Gal-1 were detected in infected brains (18).

The aquaporins (AQPs) are a family of 13 hydrophobic integral transmembrane water channel proteins involved in transcellular and transepithelial water movement and fluid transport. AQPs especially AQP4 — are implicated as proinflammatory features of astrocytes. Notably, increased AQP1, AQP4, and AQP9 levels were found in the brain tissues of several scrapie infected mouse models in a time-dependent manner. AQP1 levels are increased in the cortex regions of some human PrDs. AQPs-positive cells are astrocyte-like morphologically and co-localize with GFAP positive proliferative astrocytes. Areas predominant with AQPs overlap with abundant PrP<sup>Sc</sup> in brain tissues in scrapie murine models, strongly reflecting active neuroinflammation in prion disease (*19*).

## DEGRADATION OF ABNORMAL PrP MUTATNS AND PrP<sup>Sc</sup> IN CELLS

Hsp70 levels are increased in the brains of prion infected hamsters. Hsp70 can form complexes with abnormal Cyto-PrP and PG14-PrP that accumulates in cytoplasm, but not with wild-type PG5-PrP. Overexpression or activation of Hsp70 selectively mediates degradation of Cyto-PrP and PG14-PrP and

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reduces cytotoxicity (20). Another heat shock protein, Hsp104, present in cellular mitochondrion of fungus, bacteria, and plants, has an effect on biochemical features of PrP *in vitro*. The recombinant yeast Hsp104 can inhibit fibril assembly of the synthetic PrP106-126 peptide and formation of fibril-like structure. Treatment of Hsp104 shifts secondary structures of PrP106-126 fibrils from  $\beta$ -sheet to random coil. Hsp104 disassembles mature PrP106-126 fibrils (21).

Protein p62/sequestosome 1 (SQSTM1) is a key adaptor involved in autophagy-lysosome cargo p62/SOSTM1 degradation. Overexpression of efficiently relieves cytosolic PrP (Cyto-PrP and PG14) aggregations and cell apoptosis. Inhibition of autophagy-lysosome blocks p62/SQSTM1-mediated degradation of abnormal PrPs. More complexes of p62/SQSTM1 and LC3 in cells expressing misfolded PrPs imply that p62/SQSTM1 plays an important role in the homeostasis of abnormal PrPs via an autophagy-lysosome-dependent way (22).

Polo-like kinase 3 (PLK3) has been proven to interact molecularly with PrP in vitro and in vivo. Overexpression of PLK3 significantly decreases levels of cytosolic mutated PrPs (Cyto-PrP and PG14) in cultured cells, but does not affect levels in wild-type PrP. The kinase domain of PLK3 appears to be responsible for clearance of abnormal PrPs independently of its kinase activity. Knockdown of PLK3 aggravates the accumulation of cytosolic PrPs. PLK3 overexpression in a scrapie infected cell line causes a notable reduction of PrPSc levels, but with little effect on PrP<sup>c</sup> expression in its normal partner cell line, SMB-PS. Recovery of PLK3 at early stages of prion infection may help prevent toxic accumulation of  $PrP^{Sc}$  in brain tissues (23).

Overexpression of PLK3-mediated degradation of PrP mutants and  $PrP^{Sc}$  is repressed by lysosome inhibition. PrP mutants can interact with two major components of chaperone-mediated autophagy (CMA) effectors: lysosome-associated membrane protein type 2A (LAMP2a) and heat shock cognate protein 70 (Hsc70). Overexpression of PLK3 significantly enhances cellular levels of LAMP2a and Hsc70, accompanied with a reduction of accumulations of mutant PrP and PrP<sup>Sc</sup>. Time-dependent reduction of LAMP2a and Hsc70 has been observed in brain tissues of prion infected hamsters, indicating impairment of CMA during prion infection (Figure 2) (*24*).

#### PERSPECTIVE

Our numerous studies propose a double-edged sword of activation of brain innate immunity and neuroinflammation in the progression of prion disease neuroprotective and neurotoxic outcomes. Although there are often conflicting results from different studies regarding specific immunoelements, it is generally considered that activation of innate immunity, probably at an early stage, facilitates clearance of pathogenic prions and maintenance of normal neuronal biology. Long-term overactivation of brain innate immunity may exceed neuron tolerance thresholds at some point in time, leading to persistent and irreversible damage. Precise intervention of local immunoresponses at correct time points is an important principle for guiding R&D of immunotherapy for prion diseases.

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## Extensive Disturbances of Intracellular Components and Dysfunctions of Biological Pathways in the Brain Tissues During Prion Infection — China's Studies

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## ABSTRACT

The study describes some of the major findings of changes in intracellular components and biological pathways in the brain during prion infection and hypothesizes some important physiological and pathological approaches mainly based on our studies. Omics techniques analysis of messenger RNA (mRNA) and proteins were carried out in the study. Meanwhile, Western blot, immunohistochemistry, and immunofluorescence were used for protein analysis in different signaling pathways. Statistical analyses were used to describe the protein differences in signaling pathways of infected and normal samples. This report reviewed and summarized our studies on the aberrant changes in intracellular components and biological functions in the brains of prion disease (PrD). Omics analyses proposed extensive abnormal alterations of brain mRNAs transcriptions, protein expressions, and post-translational modifications. The molecular disturbances for microtubule instability and depolymerization, the dysregulations of different signals related with neuron loss and synaptic plasticity, the abnormalities of mitochondrial and endoplasmic reticulum stress, and disturbance of intracellular reactive oxygen species homeostasis during prion infection were precisely analyzed and reviewed. Aberrant disturbances of numerous biological molecules and signals in brain tissues were found during prion infection.

Prion disease (PrD) belongs to neurodegenerative diseases showing many similarities to Alzheimer's disease (AD) and Parkinson's disease (PD). Long incubation time and short clinical duration of PrDs highlight a slow progression at the early stage and rapid deterioration at the late stage of various abnormalities in central nerve system (CNS). In this report, we briefly describe some of the major findings of changes in intracellular components and biological pathways in the brain during prion infection including microtubule-associated proteins (MAPs), microtubule affinity-regulating kinase 4, p21-activated kinases, CK2, Polo-like kinases, etc. as well as hypothesizes some physiological and pathological approaches mainly based on our studies.

## EXTENSIVE DISTURBANCES OF GLOBAL BRAIN GENE TRANSCRIPTIONS, TRANSLATIONS AND POST-TRANSLATIONAL MODIFICATION

The similarities in global brain differentiallyexpressed mRNAs (messenger RNA) and in the biological pathways have been found in different types of human PrDs, such as sporadic Creutzfeldt-Jacob disease (sCJD), fatal familial insomnia with D178N mutation (D178N FFI) and genetic CJD with G114V mutation (G114V gCJD) (1-3). The commonly changed pathways involved signal transduction, ion transport, oxidative phosphorylation, regulation of actin cytoskeleton, MAPK signaling pathway, etc.

Global brain protein profiles in sCJD, FFI, and G114V cases revealed comprehensive disturbances at the terminal stage (4). Many dysregulated biological pathways were commonly identified, such as oxidative phosphorylation, protein export, drug metabolism cytochrome P450, PPAR signaling pathway, and fatty acid metabolism. Analysis of middle and terminal stages of two scrapie agents (139A and ME7) infected mouse models verified more differentially expressed

proteins and involved pathways at the final stage. Great similarity in differentially expressed proteins, affected pathways, and biological functions were observed between scrapie-infected mice and human sCJD (5).

Proteomic assays of global S-nitrosylation (Snitrosoproteome) in brain tissues of the patients with sCJD, FFI, and G114V gCJD (6) revealed that sCJD contained much more differentially expressed SNOproteins (DESPs), while FFI had less numbers of DESPs. The most affected pathways were PD, AD, Huntington's disease, arginine and proline metabolism, and systemic lupus erythematosus. Further study found that the brain levels of nitric oxide (NO) and nitric oxide synthase (NOS) activities of scrapie rodent models started to increase at early stage, reached the peak in the middle stage, and dropped down at late stage (7), which were closely associated with the similar alternative tendencies of many brain proteins.

Proteomic mass spectrometry of global profiles of brain acetylated proteins of scrapie-infected mice showed that overwhelming majority of the differentially expressed acetylated proteins (DEAPs) in mid-early stage was down-regulated, while more portions of DEAPs in mid-late and late stages were upregulated. Approximately 22.1% of acetylated peptides were mitochondrial-associated. Carbon metabolism, metabolic pathways, biosynthesis of amino acids, glycolysis/gluconeogenesis, pyruvate metabolism, and tricarboxylic acid (TCA cycle) were commonly affected pathways. Overall, 2 major elements in the Sirtuin (Sirt) family, Sirt 1 and Srit 3 were markedly decreased (8-9), which subsequently caused increases of acetylated forms of many down-stream target proteins, such as p53, PGC-1 and STAT3, as well as SOD and ATP5  $\beta$ , and influenced cell viability, scrapie-like prion protein (PrP<sup>Sc</sup>) deposit, cellular ROS level and mitochondria function remarkably.

## MICROTUBULE INSTABILITY AND DEPOLYMERIZATION

Our serial studies proposed that microtubule disturbance was a common phenomenon in prion infection (Figure 1). In *in vitro* assays, prion protein (PrP) revealed actively molecular interaction with tubulin, with the main interacting region within PrP in the domain of five octapeptide repeats (ORs). The wild-type PrP could efficiently stimulate microtubule assembly *in vitro* and antagonize Cu<sup>2+</sup>-induced microtubule-disrupting activity *in vivo* (10). The gCJD-related PrP mutants with inserted or deleted ORs showed much stronger inhibitive capacities on the microtubule dynamics (11). Expression of Cyto-PrP, a



FIGURE 1. Schema of prion-induced microtubule instability and depolymerization in brains.

Note: PrP<sup>sc</sup> and PrP mutants accumulate in neurons and induce the abnormal changes in tubulin, calpain, and many MAPs, leading to microtubule instability and depolymerization.

Abbreviation: MAPs=microtubule associated proteins; PrP<sup>sc</sup>=scrapie-like prion protein; PrP=prion protein, TPPP=Tubulin polymerization promoting protein.

truncated form of PrP removal of its signal peptide and GPI anchor in cultured cells induced remarkable disruption of microtubular structures (12). Knockdown of the expression of PrP via RNA interference antagonized the cytotoxicity of gCJD-associated PrP mutants in cultured cells (13).

PrP regulates microtubule dynamics via interacting with microtubule-associated proteins (MAPs). Tubulin polymerization promoting protein/p25 (TPPP/p25) is a brain-specific protein stabilizing cellular microtubular ultrastructure. The brain levels of TPPP of scrapieinfected hamsters were significantly reduced. Distinct molecular interactions between TPPP and PrP were identified. Expression of TPPP in cultured cells significantly antagonized the disruption of microtubule structures and rescued the cytotoxicity caused by the accumulation of Cyto-PrP (*14*).

Tau proteins are also important MAPs in the CNS, showing molecular binding capacity with PrP (15). Tau can interact with tubulin and facilitate microtubule formation. *In vitro*, PrP showed a notable inhibitive effect not only on the interaction of tau with tubulin, but also on the tau-mediated microtubule formation (16). GSS-related mutant P102L and gCJD-related mutants with 2 and 7 extra ORs showed more active binding capacity with tau than wild-type PrP (17). Inhibition of PrP on tau-enhanced formation of microtubule undergoes, at least partially, through blocking the interaction between tau and microtubule.

Microtubule affinity-regulating kinase 4 (MARK4) is responsible for phosphorylating neuronal MAPs. Significant decreases of brain MARK4 were found in human CID and scrapie-infected experimental animals, which showed a close correlation with the amounts of PrPSc. Treatment of toxic PrP106-126 peptide onto cultured cells induced a similar reduction of MARK4 and reduction of p-tau at Ser262, indicating that a reduction of MARK4 will result in abnormalities of tau phosphorylation and possibly induce further detachment of microtubules and hinder microtubule transportation (18). We have also identified that MAP2 was markedly decreased in the brains of human prion diseases and scrapie infected rodents. Exposure of the cultured cells to PrP106-126 peptide resulted in a significant reduction of cellular MAP2 and disrupted the microtubule structure. Meanwhile, the level of calpain, which mediates the degradation of a range of cytoskeletal proteins, was significantly increased during prion infection (19).

Protein kinase CK2 is generally composed of 2 catalytic subunits (CK2  $\alpha$  or CK2  $\alpha$ ') and 2 regulatory

subunits (CK2  $\beta$ ) and forms a tetrameric structure. The brain levels of CK2  $\alpha$  and CK2  $\beta$  decreased in the scrapie-infected experimental rodent models and human PrDs at the terminal stage (20). Strong molecular interaction was observed between PrP and CK2  $\alpha$  subunit (21). Reductions of tubulin and disruptions of microtubule structures induced by abnormal PrP mutants could be completely restored by overexpressing CK2  $\alpha$  and CK2  $\beta$  in cells. Strong binding activity of the PrP mutants with CK2 will directly affect the interaction of CK2 with tubulin and other substrates (22).

## DISTURBANCE OF THE SIGNALING RELATED WITH NEURON LOSS AND SYNAPTIC PLASTICITY

The p21-activated kinases (PAKs) are a group of protein serine/threonine kinases; among them, PAK1 and PAK3 are highly expressed in brain tissues. Remarkable reductions of PAK3 and PAK1 were detected in scrapie-infected brains. The upstream activator Rac/cdc42 and downstream substrate Raf1, particularly the phosphorylated Raf1, were remarkably decreased at terminal stage, strongly highlighting correlation of dysfunction of Rac/cdc42-PAKs-Raf1 signaling with neuron loss during prion infection (*23*).

Wnt/ $\beta$ -catenin signaling is critical for synaptic disassembly and neuron loss. In prion infected rodent models, the brain levels of phosphor- $\beta$ -catenin (Ser<sup>33,37</sup> and Thr<sup>41</sup>) were significantly up-regulated and its target substrate cyclin D1 was down-regulated. Increases of dickkopf-1, the antagonist of Wnt/ $\beta$ catenin signaling, and decreases of Wnt-3 and phosphor-GSK3  $\beta$  were also found in scrapie-infected brains. Such abnormal changes displayed timedependent progression along with the incubation period, reflecting a repression of Wnt/ $\beta$ -catenin signaling in neurons (24).

Polo-like kinases (PLKs) are a family with five major members. PLK3 phosphorylates Cdc25C on Ser<sup>216</sup> and inhibits the activity of Cdc25C, while PLK1 phosphorylates Cdc25C on Ser<sup>198</sup> and leads to activation and nuclear import, which is vital in synaptic plasticity. The increase of PLK1 and decrease of PLK3 were identified in scrapie-infected brains. Cdc25C and its phosphorylated forms were downregulated, and Cyclin B1 and PCNA were obviously up-regulated. Aberrant changes of cell cycle regulatory PLKs-Cdc25C-Cyclin B1signaling in the prioninfected neurons lead to the cell cycle arrest at M phase (25).

Brain-derived neurotrophic factor (BDNF) appears primarily presynaptic effects at central, autonomic, and neuromuscular synapses. The brain level of BDNF was significantly reduced in scrapie-infected hamsters (*26*). The major components of BDNF signaling were also remarkably decreased, including tropomyosin-related kinase (Trk) receptors, phosphor-TrkB (p-TrkB), growth factor receptor-bound protein 2 (GRB2) and neurotrophin receptor p75 (p75NTR). Remarkable reduction of brain BDNF-TrK-GRB2-p75NTR signaling reflects a severe depletion of neurotrophic factors and a defect of energy metabolism in local microenvironment.

Nerve growth factor (NGF) is another member of neurotrophin family. A decrease of brain NGF was observed in the prion infected experimental animals and in prion infected cell line (27–28). Its upstream positive regulatory kinases, p-CREB, p-CaMKIV and CaMKK2, were decreased, whereas the negative regulatory one, p-CaMKK2, was increased. The aberrant situations of those kinases in prion infected cell lines could be also partially reversed by removal of prion agent by resveratrol. Prion infection affects the activity of CDK5-CaMKK2-CaMKIV-CREB cascade, which reduces the expression of NGF.

Glucose transporter 3 (GLUT3) mainly mediates the glucose passing through the membrane of neurons. The brain GLUT3 levels in scrapie-infected rodents and the prion infected cell line were significantly downregulated (29). The level of hypoxia-inducible factor-1 alpha (HIF-1  $\alpha$ ), which positively regulated the expressions of GLUTs, was also markedly downregulated in the scrapie-infected brains. The glucose uptake activity in the prion infected cell line was markedly decreased. It indicates a severe defect in glucose uptake and metabolism of neurons during prion infection.

## MITOCHONDRIA AND ER STRESS AND DISTURBANCE OF INTRACELLULAR ROS HOMEOSTASIS

Abnormal mitochondrial, oxidative and ER related stresses were observed in the prion infected brains and the cultured cells accumulated with  $PrP^{Sc}$  or PrP mutants, involving numerous brain proteins and pathways (Figure 2). Reliable molecular interaction between PrP and 14-3-3  $\beta$  *in vitro* and *in vivo* was



FIGURE 2. Schema of prion-induced mitochondrial stress, ER stress, and oxidative stress in brains. Note: Accumulations of PrP<sup>sc</sup> and PrP mutants cause aberrant alterations (either expressing level or activity or translocation) of numerous brain proteins and pathways related to mitochondrial stress, ER stress, and oxidative stress. Abbreviation: PrP<sup>sc</sup>=scrapie-like prion protein; PrP=prion protein; ER=endoplasmic reticulum. illustrated. Wild-type PrP was promoted, but PrP106-126 peptide inhibited the dimerization of 14-3-3  $\beta$ (*30*). Time-dependent reduction of 14-3-3 was detected in the brains of scrapie-infected mice, accompanied with the increase of its *S*-nitrosylated form and many mitochondrial apoptotic agents. Challenge of PrP106-126 onto the cultured cells decreased the level of 14-3-3 and induced translocations of cellular Bax to the membrane fractions. Knockdown of 14-3-3 aggravated the mitochondrial apoptosis in PrP106-126 exposed cells (*31*).

Increases of ER-stress-related proteins Grp94, XBP1, TRAF2, and CHOP in the cells expressing PrP mutants with extra ORs (PG9 and PG12), while decrease of Bcl-2 and increase of cytochrome C in the cells expressing the mutant removal of ORs (PG0). The mutants with inserted ORs underwent mainly via ER stress and the mutant without OR via mitochondrial-related pathway (32). Accumulation of Cyto-PrP in cytoplasm also induces cell apoptosis, in which mitochondrial apoptotic pathway seems to be essential (33). Dynamin-related protein 1 (Drpl) and optic atrophy protein 1 (Opa1) are two essential elements for mitochondria fission and fusion. Reductions of brain Drp1 and Opa1 were noticed in scrapie-infected animals, indicating various disturbance of brain mitochondria dynamics (34).

Swelling mitochondria structures were observed in the prion-infected cell line by transmission electron microscopy. The levels of Pink1 and Parkin, particularly in the mitochondrial fraction, were increased, whereas the levels of mitochondrial membrane proteins TIMM44, TOMM20, and TIMM23 were decreased. The brain Pink1 and Parkin in mice infected with scrapie were also increased at the terminal stage, predominantly located at the areas with more PrP<sup>Sc</sup> deposit. Enhanced Pink-Parkin pathway mediated activated mitophagy during prion infection (*35*).

Cells expressing PrP mutants (KDEL and 3AV) produced an 18-kD large C-terminal proteolytic resistant fragment (Ctm-PrP). Those cells were more sensitive to ER stress stimuli, showing ER-mediated apoptosis by CHOP and the caspase-12 apoptosis pathway. Ctm-PrP and ER stress were only observed in the cells expressing PrP mutants in the transmembrane region (G114V and A117V), but not in those expressing the mutants outside the transmembrane region (*36*). Protein disulfide isomerase (PDI) is an enzymatic chaperone for reconstructing misfolded protein in ER lumen. In the scrapie-infected hamsters, abnormally upregulated brain PDI, Grp78 and Grp58 were detected. Expressions of PrP mutants in the cultured cells induced high levels of PDI and Grp18 and cytotoxicity. PDI and its relevant executors may function as a pleiotropic regulator in the processes of misfolded PrP proteins (*37*).

The OR domain was critical for PrP antioxidation. Disruption of ORs induced a high level of ROS and a low level of glutathione peroxidase in cells that were more susceptible to the challenges of oxidative factors (38). Abnormal increases of ROS are repeatedly reported in the brains of human and animal prion diseases. Molecular interaction between PrP and HS-1 associated protein X-1 (HAX-1), a protein associated with cell apoptosis. Co-expression of wild-type PrP and HAX-1 increased the cultured cells to resist the challenge of H<sub>2</sub>O<sub>2</sub> (39).

## PERSPECTIVE

Although the clinical and general neuropathological features of prion disease have been well documented for a hundred years, the molecular abnormalities started to be revealed in the past three decades. Along with the development and progress of molecular biology and omics, the mysterious veil of the pathogenesis of CJD-associated neuropathological changes has been gradually revealed by uncovering the aberrant alterations of dozens of bioactive components and biological pathways. Extensive dysfunctions of numerous biological processes and signals directly lead to slow but irreversible damage on neurons and brain tissues infected with prions, the majority of them occurring much earlier than the appearance of clinical symptoms, even earlier than the appearance of detectable PrPSc. Other neurodegenerative diseases, as AD and PD, also display such similar neuropathological processes. However, besides the unique infectivity of prions, the clinical duration of PrD is much shorter, which possibly reflects a much faster and more severe neuropathological deterioration.

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