Retrospective Analysis of Sheep Scrapie by Western Blotting with Formalin-Fixed Paraffin-Embedded (FFPE) Tissues

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ABSTRACT. An abnormal isoform of prion protein (PrP^{Sc}) was extracted from formalin-fixed paraffin-embedded (FFPE) tissues from sheep and analyzed by western blotting. PrP^{Sc} immunoreactivity against anti-PrP monoclonal antibody T2, which recognizes discontinuous PrP sequences, differed amongst individual scrapie sheep cases. This may reflect structural differences in PrP^{Sc} that have been formalin-fixed prior to their extraction. This study indicates that western blotting by using FFPE tissues is useful for the retrospective analysis of transmissible spongiform encephalopathies in which only formalin-fixed samples are available and in conducting transmissible spongiform encephalopathies surveillance where freezing system is insufficient.

KEY WORDS: FFPE, prion, PrP^{Sc}, scrapie, WB.

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Transmissible spongiform encephalopathies (TSEs) are neurodegenerative disorders that affect humans and animals. Bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, and Creutzfeldt-Jakob disease in humans are examples of disorders included in this category [15]. Abnormal isoforms of prion proteins (PrP^{Sc}) are major components of pathogenesis [15], and the detection of PrP^{Sc} is the key for TSE diagnosis. Western blotting (WB) and immunohistochemistry (IHC) are routinely used as confirmatory tests. Although WB is a convenient biochemical technique for the detection of PrP^{Sc}, it is not always used because it requires frozen or fresh tissue samples.

Scrapie is caused by many different prion strains, which are characterized by their incubation periods and neuropathology in inbred mice [5]. Strain-specific properties are attributed to the different conformations of PrP^{Sc} [1, 2, 13, 18]. The molecular profiles of PrP^{Sc} in WB, including the glycoprofiles and molecular weights of proteinase-K (PK)-digested PrP^{Sc} (PrP^{Sc}core), are used for the classification of prion strains [3]. As such, it is known that the PrP^{Sc} of BSE and that of scrapie in sheep can be distinguished using WB [14].

In Mongolia, sheep and goats are the major livestock, with hide, skin, cashmere, and meat exports increasing every year. Because there have been no outbreaks of scrapie in Mongolia to date, it is of crucial importance that Mongolian sheep and goats are confirmed negligible of scrapie infec-

tion through consistent surveillance. Unfortunately, it is often difficult to collect and transport frozen brain tissues to laboratories for surveillance, which is also an issue experienced by BSE-affected countries. This is primarily because of geography, with Mongolia being a vast extent of land that includes both deserts to mountainous regions that can be subject to harsh climatic conditions. Nonetheless, pathological examinations are routinely used for the diagnosis of contagious diseases, including rabies and listeriosis.

It has been reported that PrP^{Sc} extracted from formalin-fixed paraffin-embedded (FFPE) tissues can be detected by WB [12]. Because this technique could enable better TSE surveillance, we tested its usefulness on tissues from sheep scrapie cases for which frozen tissues were unavailable. We have demonstrated that WB analysis by using FFPE will be useful for retrospective analysis of transmissible spongiform encephalopathies.

Brain samples containing scrapie Chandler [7], 79A (TSE Resource Center, Compton, UK), Kanagawa [10], and mouse-adapted BSE [6] affected wild-type mice (ICR) were used. Samples were either stored at –80°C for biochemical analysis, or fixed in 10% buffered formalin solution and embedded in paraffin for histopathological and immunohistochemical examination. Fixed tissues were treated with 98% formic acid for 60 min to reduce their infectivity [17] prior to embedding. Furthermore, 4 FFPE tissues of natural sheep scrapie cases were also examined (Table 1).

For the purification of PrP^{Sc} from frozen tissues, brains were homogenized in a buffer containing 100 mM NaCl and 50 mM Tris-HCl (pH 7.6). The homogenate was mixed with an equal volume of detergent buffer containing 4% Zwittergent 3–14, 1% sarkosyl, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.6) and then incubated with 0.25 mg collagenase. The homogenate was then incubated with 40 µg/ml of PK at

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Table 1. PrP^{Sc} detection from FFPE tissues of scrapie sheep

No.	Sheep breed	Year	Scrapie diagnosis		PrP ^{Sc} detection from FFPE tissues		Remarks
			WB	IHC	mAb T2	mAb SAF84	
1	Corriedale	1991	ND ^{a)}	+	±	++	Field case at Fukushima, Fig.2, lane 1
2	Suffolk	1994	ND	+	±	+++	Field case at Hokkaido-1, Fig. 2, lane 2
3	Suffolk	1994	ND	+	±	+++	Field case at Hokkaido-2, Fig. 2, lane 3
4	Suffolk	1999	ND	+	++	+++	Field case at Tokyo, Fig.2, lane 4

a) Not done (no frozen sample was available).

Fig. 1. PrP^{Sc} analysis of frozen tissues (A) and FFPE tissues (B) by using western blotting. A. PrP^{Sc} extracted from frozen tissues was detected using mAbs T2 (left panel) and SAF84 (right panel). B. PrP^{Sc} extracted from FFPE tissue was detected using mAbs T2 and SAF84. Lane 1, mouse-adapted BSE-affected ICR mice; lane 2, Scrapie Kanagawa strain-affected ICR mice; lane 3, mouse-adapted scrapie 79A strain-affected ICR mice. This sample was examined without formic acid treatment. Lane 4, mouse-adapted scrapie Chandler strain-affected ICR mice. C. Lanes 5–7, FFPE tissues from normal mouse brains. Lane 8, PrP^{Sc} from mouse-adapted scrapie (frozen tissue).

37°C for 30 min. PK digestion was terminated using 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc; Roche Diagnostics). The sample was mixed with 2-butanol:methanol (5:1) and centrifuged at $20,000 \times g$ for 10 min. The final pellet was analyzed using WB.

The protocol for PrP^{Sc} extraction from FFPE tissues was followed as reported previously [8] with minor modifications. Briefly, a section from each FFPE tissue block (PET block) was collected into a 1.5-ml microcentrifuge tube. To each tube, 150 μ l of buffer (50 mM Tris, 1 mM EDTA and 0.5% Tween 20, pH 7.5) was added. The tube was incubated at 100°C for 10 min and was then immediately frozen with cold ethanol for 5 min. This process was repeated once, and the tubes were then incubated for additional 10 min at 100°C. The sample was then immediately centrifuged at $20,000 \times g$ for 15 min to separate the paraffin from the buffer. The tissue pellets and aqueous layer were transferred to another tube, and 150 μ l of detergent buffer containing 4% Zwittergent 3–14, 1% sarkosyl, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.6) was added. The pellet was dispersed by sonication and mechanical shearing by using a 27-gauge needle. The sample was then mixed with PK to a final concentration of 50 μ g/ml and incubated at 37°C for 30 min. Digestion was stopped by adding 1 mM of Pefabloc. The sample was mixed with 2-butanol:methanol (5:1) and centrifuged at $20,000 \times g$ for 10 min. The final pellet was analyzed using WB as described previously [9]. The PrP signal was detected using either anti-PrP mAb SAF84 (SPI-Bio, Montigny le Bretonneux, France) and T2 [16], which react against epitopes 160–170 of human PrP sequences and 163–168 of murine PrP sequences, respectively.

WB analysis of PrP^{Sc} from frozen tissues of wild-type mice is shown in Fig. 1A, and the 2 mAbs showed a typical PrP^{Sc} banding pattern. PrP^{Sc} was detected in all the FFPE tissue samples that were examined using both mAbs (Fig. 1B). The molecular weight of PrP^{Sc} from BSE-affected wild-type mice was lower than that of samples from scrapie (Fig. 1, lane 1). Although the molecular weights of PrP^{Sc} from FFPE tissues were consistent with those of PrP^{Sc} from frozen tissues for both T2 and SAF84, the glycoform profile of PrP^{Sc} from FFPE tissues was resembled but not consistent with that of frozen tissues (Fig. 1A). A ~14 kDa PrP^{Sc} fragment was detected using mAb SAF84 in the FFPE tissue of Chandler-affected mice (Fig. 1B, lane 4), but this truncated fragment was not detected during the analysis of frozen tissues (Fig. 1A, lane 4). Formalin fixation influenced PK

Fig. 2. Comparison of immunoreactivity of PrP^{Sc} extracted from FFPE tissue of natural scrapie A. PrP^{Sc} extracted from FFPE tissue of natural scrapie was detected using mAb T2. B. The same membrane was exposed longer time to visualize the unglycosylated PrP^{Sc} band. C. The same sample was analyzed using mAb SAF84. Lane 1, field scrapie case of 1991; lanes 2 and 3, field scrapie case of 1994; lane 4, field scrapie case of 1999; lanes 5 and 10, PrP^{Sc} extracted from typical scrapie sheep brain (frozen tissue); lane 6, PrP^{Sc} from BSE-affected cattle brain (frozen tissue); lanes 7–9, TSE-negative sheep brain samples.

digestion of PrP^{Sc} and producing some PrP^{Sc} in a partially digested truncated form. Furthermore, strong signals of ~40 kDa were detected for all the PrP^{Sc} from the FFPE tissues that were examined using SAF84 (Fig. 1B). Formalin fixation induces alkylation of amino residues and cross-linking between bio-molecules. Though the precise mechanisms are obscure, these phenomena may cause by this cross-linking. No PrP signals, either low- or high-molecular weight, were detected in TSE-negative FFPE tissue samples (Fig. 1C).

Then, we examined the PrP^{Sc} detection in FFPE tissues from natural sheep scrapie. PrP^{Sc} was extracted from FFPE tissue samples obtained from 2 breeds of sheep having scrapie (Suffolk and Corriedale). These samples were fixed between 1991 and 1999 and stored at room temperature. The PrP^{Sc} signal was clearly detected in 1 sample (Case No. 4), while a weak positive signal was detected in the other 3 samples (Case Nos. 1–3) by using mAb T2 (Table 1, Fig

2A). In contrast, all the examined samples showed a strong positivity for mAb SAF84 (Table 1, Fig. 2C). The molecular weights of the PrP^{Sc} from the 4 natural scrapie cases were similar to those of typical scrapie (Fig. 2B). No PrP signal was detected in the FFPE tissue samples obtained from healthy sheep (Fig. 2).

We have shown that PrP^{Sc} can be extracted from FFPE tissues [8, 11]. In this study, we used 2 different mAbs. PrP^{Sc} from FFPE tissues of scrapie sheep have different immunoreactivities against mAb T2, which recognizes discontinuous sequences at PrP163–168 [16]. We concluded that the different immunoreactivities of PrP^{Sc} in FFPE tissues were not associated with the sheep breed or sample storage time but may be potentially associated with their conformational differences. Our results showed the possibility that immunoreactivity of PrP^{Sc} from FFPE tissues might be associated with prion strain diversities in sheep.

The molecular weights of all the PrP^{Sc} extracted from FFPE tissues of natural scrapie cases were similar to those of typical scrapie but different from those from BSE (Fig. 2B). This result indicates that these scrapie cases occurred independently of BSE; however, it is important to note that the origin of BSE has not been identified and sheep scrapie has been considered as a candidate of origin. Furthermore, the potential presence of BSE in small ruminants is cautioned, and a BSE case in a goat has been reported [4]. To investigate the relationship between BSE and scrapie, characterization of individual prions is necessary.

This technique could detect PrP^{Sc} from FFPE tissues that were kept more than 10 years. Our study showed that WB analysis by using FFPE will be useful for retrospective analysis in which only formalin-fixed samples are available and in conducting TSE surveillance where freezing system is insufficient.

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