NOTE *Pathology*

Coexistence of two forms of disease-associated prion protein in extracerebral tissues of cattle infected with H-type bovine spongiform encephalopathy

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ABSTRACT. H-type bovine spongiform encephalopathy (H-BSE) is an atypical form of BSE in aged cattle. H-BSE is characterized by the presence of two proteinase K-resistant forms of disease-associated prion protein (PrP^{Sc}), identified as PrP^{Sc} #1 and PrP^{Sc} #2, in the brain. To investigate the coexistence of different PrP^{Sc} forms in the extracerebral tissues of cattle experimentally infected with H-BSE, immunohistochemical and molecular analyses were performed by using N-terminal-, core-region- and C-terminal-specific anti-prion protein antibodies. Our results demonstrated that two distinct forms of PrP^{Sc} coexisted in the various extracerebral tissues.

KEY WORDS: atypical BSE, extracerebral tissue, H-type, peripheral nervous system, prion

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Bovine spongiform encephalopathy (BSE) belongs to a group of prion or transmissible spongiform encephalopathy diseases and is a fatal, progressive degenerative disorder of the central nervous system (CNS) in cattle. The disease is characterized by the development of spongiform changes and accumulation of an abnormal isoform of a prion protein (PrP^{Sc}) that is thought to represent a post-translational modification of the normal, host-encoded cellular prion protein (PrP^{C}) principally found in the CNS of affected hosts [22]. Currently, BSE is classified into at least three different strains comprising food-borne-related or epidemic BSE, named classical BSE, and putatively sporadic forms of H- or L-type atypical BSEs on the basis of the molecular mass of the proteinase K (PK)-resistant PrP^{Sc} [3, 6].

According to western blot (WB) analyses, the molecular characteristics of H-BSE isolates from cattle consist of two forms of PK-resistant PrP^{Sc}: PrP^{Sc} #1 and PrP^{Sc} #2 [4, 11]. Since glycosylated C-terminal PK-resistant PrP^{Sc} #2 results from a C-terminal truncation at a position between amino-acid residues Ser154 and Glu163 of bovine PrP following PK digestion, detection of PK-resistant PrP^{Sc} #1 is possible using either N-terminal-specific antibodies, such as P4 (bovine PrP epitope $_{101}$WGQGGSH$_{107}$), or core-region-specific antibodies, such as F89/160.1.5 ($_{148}$PLIHFGSD$_{155}$), 12F10 ($_{154}$SDYEDRYYRE$_{163}$) and 6H4 ($_{155}$DYEDRYYRE$_{163}$). After deglycosylation, the unglycosylated form of PK-resistant PrP^{Sc} #1 was detected as a 19 kDa molecule, which was capable of interactions with various anti-prion protein (PrP) antibodies, while that of PK-resistant PrP^{Sc} #2 was detected as a 10−12 kDa fragment recognizable by C-terminal region-specific antibodies, such as SAF84 ($_{175}$RPVDQY$_{180}$) or F99/97.6.1 ($_{229}$YQRE$_{232}$).

The pathologic and molecular features of experimental H-BSE in cattle have already been described [1, 9, 10, 13, 14, 19]. Previous studies revealed that minimal quantities of PrP^{Sc} accumulation could be detected in the extracerebral tissues of experimental H-BSE cattle by immunohistochemistry (IHC) using C-terminal-specific monoclonal antibodies (mAbs) [13, 15]. The different truncated forms of PrP^{Sc} depended upon the cell-specific proteolytic cleavage and cell- and tissue-specific PrP^{Sc} conformational differences [12]. However, characteristics associated with accumulated PrP^{Sc} in the extracerebral tissues of H-BSE-infected cattle remain unclear. The aim of this study was to examine the IHC-staining and molecular properties of PrP^{Sc} in cattle experimentally infected with H-BSE.

Animal experiments were approved by the Institutional Animal Care and Use Committee at the National Institute of Animal Health (approval ID: 07-9). Experimental transmission of H-BSE to cattle has previously been described (case No. 1, code 7749; case No. 2, code 9458; and case No. 3, code 0728) [19]. Two calves inoculated with 10% normal brain homogenates prepared from healthy cattle served as controls and were sacrificed at the age of 35 months. The tissue samples examined in this study were as follows: brains, spinal cords (C8 or L6), spinal nerves (C8 or L6), dorsal root ganglia (C8 or L6), cauda equina, trigeminal ganglion, retinas, optic nerves and adrenal glands. Histopathologic analysis was performed on hematoxylin and eosin (HE)-stained tissue sections. After epitope retrieval [17], the deposition of PrP^{Sc} was determined by IHC using four mAbs, namely P4 (0.2 μg/m*l*; R-Biopharm, Darmstadt, Germany), F89/160.1.5 (0.1 μg/m*l*; SPI-bio, Montigny Le Bretonneux, France), 12F10 (0.1 μg/m*l*; SPI-bio) and F99/97.6.1 (0.1 μg/m*l*; VMRD, Pullman, WA, U.S.A.), followed by administration of the TSA-biotin system (PerkinElmer, Boston, MA, U.S.A.) according to previously described protocols [16]. For WB analysis, tissue samples were dissolved in sodium

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dodecyl sulfate polyacrylamide gel electrophoresis sample buffer and assayed using standard WB procedures. PK-resistant PrP^{Sc} signals were detected with mAbs P4 (0.2 μg/m*l*), 6H4 (0.2 μg/m*l*; Prionics, Schlieren, Switzerland) and SAF84 (0.2 μg/m*l*; SPI-bio), followed by incubation with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Rockford, IL, U.S.A.) according to a previously described method [18].

In accordance with IHC results described previously [7, 18], intracellular (intraneuronal and intraglial types) and extracellular (particulate, granular, stellate and plaque-like types) PrP^{Sc} deposits were immunolabeled with mAb F99/97.6.1. However, immunolabeling intensity reflecting the detectable PrP^{Sc} in the brain and extracerebral tissues varied with the antibodies tested (Figs. 1 and 2). In general, intracellular PrP^{Sc} deposits in the Schwann cells of the cauda equina or spinal nerves, the ganglion cells and satellite cells of ganglia, the ganglion cells of the retina, and the glial cells of the optic nerve that were immunolabeled with mAb F99/97.6.1 exhibited stronger signals, while those labeled with mAb 12F10 exhibited weak to moderate signals, respectively (Fig. 2). Immunolabeling intensity and patterns of PrP^{Sc} with mAb F89/160.1 were similar to those observed with mAb 12F10 (data not shown). Additionally, granular deposits of PrP^{Sc} were detected at the periphery of axons, referred to as adaxonal PrP^{Sc} depositions, with mAb F99/97.6.1 [15], but these were not detectable using other

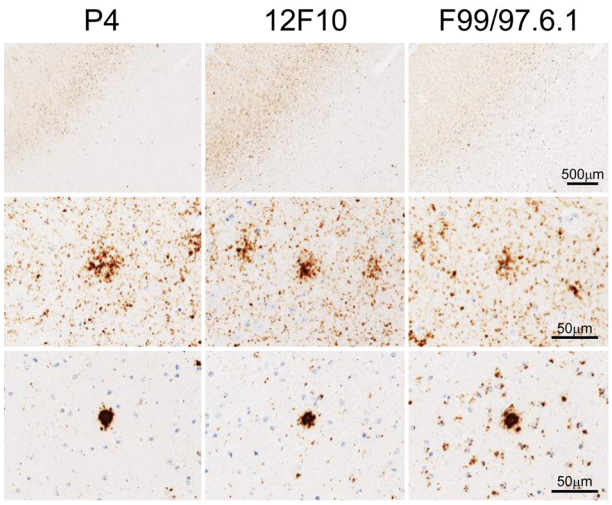

Fig. 1. Panel showing serial sections taken from the frontal lobe of cattle infected with H-BSE. Sections were immunolabeled with three mAbs (P4, 12F10 and F99/97.6.1), detected using the TSA-biotin system and counterstained with HE. The upper row indicates a low magnification image of the frontal lobe. Immunolabeled PrP^{Sc} accumulated widely in the cerebral gray matter (left side), but less in the white matter (right side). The middle row shows a high-magnification image of the frontal gray matter. The bottom row shows a high-magnification image of the frontal white matter.

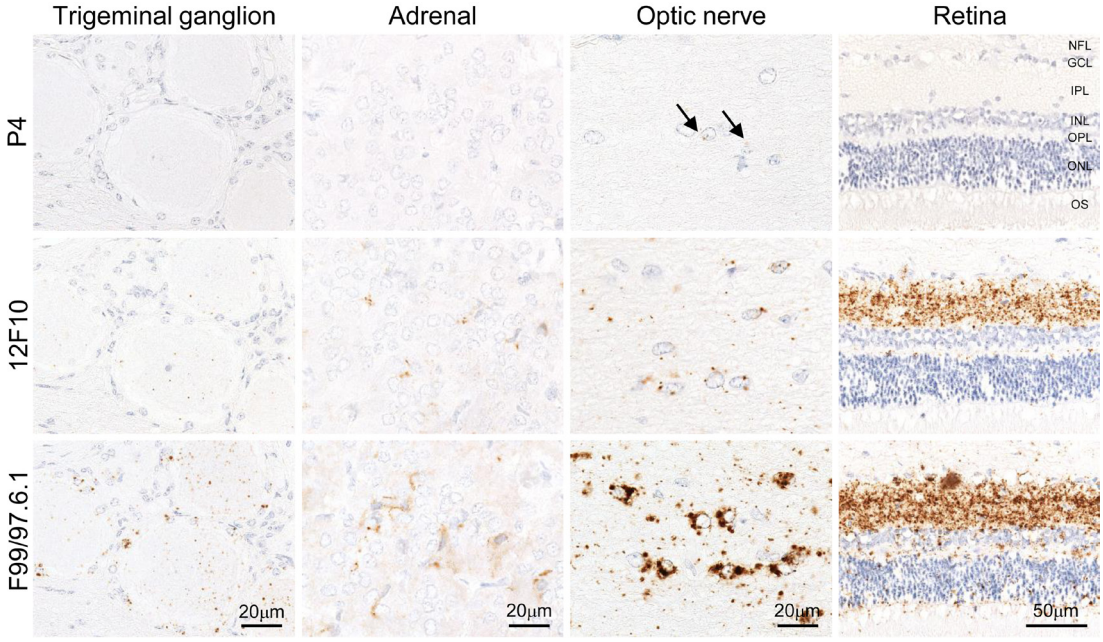

Fig. 2. Comparison of PrP^{Sc} immunolabeling intensity on serial sections of trigeminal ganglion, adrenal medulla, optic nerve and retina using three different mAbs (P4, 12F10 and F99/97.6.1), detected with the TSA-biotin system and counterstained with HE. MAb F99/97.6.1 resulted in the most intense labeling (bottom row). Only faint immunoreactivity was observed within the glial cells of the optic nerve (arrows), but no PrP^{Sc} deposits were observed in the trigeminal ganglion, adrenal and retina using mAb P4. Abbreviations in the Retina column: NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nucleus layer; OPL, outer plexiform layer; ONL, outer nucleus layer; OS, outer segments.

antibodies (data not shown). In the adrenal gland, immunolabeled PrP^{Sc} was located between chromaffin cells, which participate in intercellular processes involving sympathetic neurons (Fig. 2). In the retina, granular PrP^{Sc} accumulation was pronounced in the ganglion cell layer, as well as the inner and outer plexiform layers, using both C-terminal- and core-region-specific antibodies (Fig. 2). Interestingly, faint intraglial staining was seen with mAb P4 in the optic nerve, but mAb P4 did not yield any immunoreactions in other tissues, such as the trigeminal and dorsal root ganglia, cauda equina and spinal nerves (Fig. 2). By conventional WB analysis, all antibodies used in this study resulted in signals detected in both the brain and all extracerebral tissues (Fig. 3). However, the signal intensity with mAb P4 in the peripheral nervous tissues, i.e., the trigeminal ganglion and cauda equina, was apparently weaker than that observed with mAb 6H4. Moreover, the multiple banding patterns of PrP^{Sc} #1 and PrP^{Sc} #2 with an additional 10–12 kDa band were detected in these tissues with mAb SAF84 (Fig. 3). Additionally, a smaller 7 kDa fragment was identified in the cauda equina and optic nerve using mAb P4 (arrowhead in Fig. 3).

Infectivity of the cauda equina from case 1 was assessed by intracerebral injection into bovine PrP-overexpressing (TgBoPrP) mice as described previously [18]. Although the incubation period of TgBoPrP mice inoculated with the cauda equina from case 1 was 354.9 ± 84.5 days (n=8) and appeared to be longer than that for mice inoculated with the brain tissue from the original cattle (315.8 ± 11.6 days, n=10) [18], no significant difference was observed in the mean incubation periods analyzed using Instat3 software (GraphPad Software; La Jolla, CA, U.S.A.), indicating that the cauda equina exhibited a high level of infectivity. Additionally, the unique clinical signs, the vacuolar lesion scores in HE-stained sections, the neuroanatomic distribution patterns of immunolabeled PrP^{Sc} and the molecular features of PrP^{Sc} in the brains of TgBoPrP mice inoculated with the cauda equina were identical to those observed in TgBoPrP mice inoculated with the brain homogenate from H-BSE-infected cattle, which constituted the original cattle inoculum (data not shown) [18].

The present study demonstrated that two different forms of PrP^{Sc}, PrP^{Sc} #1 and PrP^{Sc} #2, could propagate both in the CNS and in extracerebral tissues analyzed by WB. However, IHC was unable to discriminate between PrP^{Sc} #1 and PrP^{Sc} #2 using different antibodies. The strain-specific molecular features of H-BSE characterized by the presence of an additional 10 − 12 kDa fragment detected with mAb SAF84 were maintained in the extracerebral tissues tested in this study (Fig. 3). Peripheral PrP^{Sc} accumulation is commonly thought to result from centrifugal spreading by nerve pathways during the clinical stage of the disease [2]. The presence of higher PrP^{Sc} levels in the optic nerve relative to other peripheral nervous tissues according to IHC and WB analyses could be attributed to the optic nerve being involved with the CNS rather than the peripheral nervous system. Detection of two PrP^{Sc} forms in the sympathetic terminal nerve endings of the adrenal medulla or Schwann cells of the peripheral nerves

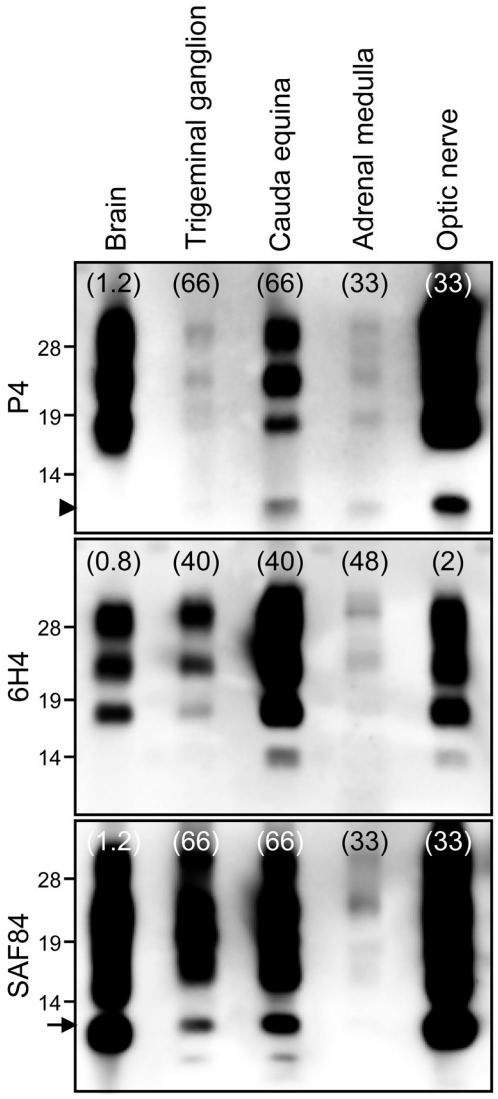

Fig. 3. Proteinase K-resistant PrP^{Sc} profiles of H-BSE prions analyzed with different mAbs (P4, 6H4 and SAF84). The arrowhead indicates a ~7 kDa fragment detected with mAb P4. The arrow indicates an additional 10–12 kDa band detected with mAb SAF84. Each number in parenthesis indicates tissue equivalent (mg) loading. Molecular markers are shown to the left (kDa).

may be attributed to a high degree of peripheral neurotropism in H-BSE [13–15, 18]. Low PrP^{Sc} levels were detected by IHC in the muscle spindles of skeletal muscles of cattle terminally affected with H-BSE [13]. In general, PrP^{Sc} levels in the peripheral tissues were believed to be much lower than those found in the brain of animals at the terminal stage of the disease.

Immunolabeling intensities of PrP^{Sc} in extracerebral tissues were different from those observed using other antibodies. The C-terminal mAb F99/97.6.1 immunolabeled a wide variety of different PrP^{Sc} stains and always returned the best results [19]. Moreover, immunoreactivity of mAb P4 for

PrP^{Sc} was apparently weaker than that of the core-specific PrP antibodies 12F10 or 6H4 according to both IHC and WB results (Figs. 1–3). Minimal levels of PrP^{Sc} were detected with mAb P4 in the optic nerve, but not in other tissues (Fig. 2). The differences in immunoreactivity observed from the N-terminal and core-specific PrP antibodies may be explained by variations in antibody affinities and immunodetection procedures, including epitope retrieval and reagents [7–12]. Since antigen-epitope retrieval might involve specific amino acids in specific locations, the chemical-based and/or autoclaving-antigen unmasking techniques used in this study could be insufficient at denaturing the PrP^{Sc} protein, which would hinder immunodetection with mAb P4 by IHC [8]. Therefore, we might exclude the possibility that mAb P4 would have a weaker affinity for PrP^{Sc} according to IHC relative to other antibodies used in this study [7].

A C-terminal 10–12 kDa fragment detected with C-terminal-specific antibodies in H-BSE may be the result of enzymatic truncation and digestion occurring at a site distant from residue 163 of bovine PrP [3, 4]. Similarly, the presence of a 10–12 kDa low molecular-weight PK-resistant C-terminal fragment in the brain was reported in various forms of Creutzfeldt-Jakob disease (CJD) in humans [5, 23, 25]. Interestingly, the C-terminal fragment was associated with non-plaque-type dura CJD [23]. In H-BSE, an additional ~7 kDa fragment detected with mAb P4, but not with mAb SAF84, by WB analysis was distinct from the 10–12 kDa fragment. This ~7 kDa fragment might be derived from the cleaved N-terminal product from the full-length PrP^{Sc}, PrP^{Sc}#1, resulting from PK digestion between amino acids 154 and 163 of bovine PrP [4, 11]. A similar finding was reported in Gerstmann-Sträussler-Scheinker disease (GSS) in humans caused by mutations of the specific PrP gene. GSS was characterized by the accumulation of PrP-amyloid plaques and the presence of an unglycosylated 8 kDa fragment cleaved from the N- and C-termini of PrPs in the brain [20, 21, 24]. Interestingly, GSS patients with only unglycosylated 8 kDa fragment accumulation were characterized by the absence of spongiform degeneration in the brain [20, 21]. These data suggested that small unglycosylated N-terminal fragments might be strongly associated with the deposition of PrP-amyloid plaques in the brain [4, 19] and the presence of aggregated PrP^{Sc} in the extracerebral tissues of H-BSE-infected animals [15].

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REFERENCES

1. Balkema-Buschmann, A., Ziegler, U., McIntyre, L., Keller, M., Hoffmann, C., Rogers, R., Hills, B. and Groschup, M. H. 2011. Experimental challenge of cattle with German atypical bovine spongiform encephalopathy (BSE) isolates. *J. Toxicol. Environ. Health A* **74**: 103–109. [Medline] [CrossRef]
2. Balkema-Buschmann, A., Fast, C., Kaatz, M., Eiden, M., Ziegler, U., McIntyre, L., Keller, M., Hills, B. and Groschup, M. H. 2011. Pathogenesis of classical and atypical BSE in cattle. *Prev. Vet. Med.* **102**: 112–117.
3. Biacabe, A. G., Laplanche, J. L., Ryder, S. and Baron, T. 2004. Distinct molecular phenotypes in bovine prion diseases. *EMBO Rep.* **5**: 110–115. [Medline] [CrossRef]
4. Biacabe, A. G., Jacobs, J. G., Bencsik, A., Langeveld, J. P. and Baron, T. G. 2007. H-type bovine spongiform encephalopathy: complex molecular features and similarities with human prion diseases. *Prion* **1**: 61–68. [Medline] [CrossRef]
5. Capellari, S., Parchi, P., Russo, C. M., Sanford, J., Sy, M. S., Gambetti, P. and Petersen, R. B. 2000. Effect of the E200K mutation on prion protein metabolism. Comparative study of a cell model and human brain. *Am. J. Pathol.* **157**: 613–622. [Medline] [CrossRef]
6. Casalone, C., Zanusso, G., Acutis, P., Ferrari, S., Capucci, L., Tagliavini, F., Monaco, S. and Caramelli, M. 2004. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. *Proc. Natl. Acad. Sci. U.S.A.* **101**: 3065–3070. [Medline] [CrossRef]
7. Gavier-Widén, D., Nöremark, M., Langeveld, J. P., Stack, M., Biacabe, A. G., Vulin, J., Chaplin, M., Richt, J. A., Jacobs, J., Acín, C., Monleón, E., Renström, L., Klingeborn, B. and Baron, T. G. 2008. Bovine spongiform encephalopathy in Sweden: an H-type variant. *J. Vet. Diagn. Invest.* **20**: 2–10. [Medline] [CrossRef]
8. Gown, A. M. 2004. Unmasking the mysteries of antigen or epitope retrieval and formalin fixation. *Am. J. Clin. Pathol.* **121**: 172–174. [Medline] [CrossRef]
9. Greenlee, J. J., Smith, J. D., West Greenlee, M. H. and Nicholson, E. M. 2012. Clinical and pathologic features of H-type bovine spongiform encephalopathy associated with E211K prion protein polymorphism. *PLoS ONE* **7**: e38678. [Medline] [CrossRef]
10. Greenlee, M. H., Smith, J. D., Platt, E. M., Juarez, J. R., Timms, L. L. and Greenlee, J. J. 2015. Changes in retinal function and morphology are early clinical signs of disease in cattle with bovine spongiform encephalopathy. *PLoS ONE* **10**: e0119431. [Medline] [CrossRef]
11. Jacobs, J. G., Langeveld, J. P., Biacabe, A. G., Acutis, P. L., Polak, M. P., Gavier-Widen, D., Buschmann, A., Caramelli, M., Casalone, C., Mazza, M., Groschup, M., Erkens, J. H., Davidse, A., van Zijderveld, F. G. and Baron, T. 2007. Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe. *J. Clin. Microbiol.* **45**: 1821–1829. [Medline] [CrossRef]
12. Jeffrey, M., Martin, S. and González, L. 2003. Cell-associated variants of disease-specific prion protein immunolabelling are found in different sources of sheep transmissible spongiform encephalopathy. *J. Gen. Virol.* **84**: 1033–1045. [Medline] [CrossRef]
13. Konold, T., Bone, G. E., Clifford, D., Chaplin, M. J., Cawthraw, S., Stack, M. J. and Simmons, M. M. 2012. Experimental H-type and L-type bovine spongiform encephalopathy in cattle: observation of two clinical syndromes and diagnostic challenges. *BMC Vet. Res.* **8**: 22. [Medline] [CrossRef]
14. Konold, T., Phelan, L. J., Clifford, D., Chaplin, M. J., Cawthraw, S., Stack, M. J. and Simmons, M. M. 2014. The pathological and molecular but not clinical phenotypes are maintained after

second passage of experimental atypical bovine spongiform encephalopathy in cattle. *BMC Vet. Res.* **10**: 243. [Medline] [CrossRef]

15. Okada, H., Iwamaru, Y., Yokoyama, T. and Mohri, S. 2013. Immunohistochemical detection of disease-associated prion protein in the peripheral nervous system in experimental H-type bovine spongiform encephalopathy. *Vet. Pathol.* **50**: 659–663. [Medline] [CrossRef]
16. Okada, H., Iwamaru, Y., Fukuda, S., Yokoyama, T. and Mohri, S. 2012. Detection of disease-associated prion protein in the optic nerve and the adrenal gland of cattle with bovine spongiform encephalopathy by using highly sensitive immunolabeling procedures. *J. Histochem. Cytochem.* **60**: 290–300. [Medline]
17. Okada, H., Sato, Y., Sata, T., Sakurai, M., Endo, J., Yokoyama, T. and Mohri, S. 2011. Antigen retrieval using sodium hydroxide for prion immunohistochemistry in bovine spongiform encephalopathy and scrapie. *J. Comp. Pathol.* **144**: 251–256. [Medline] [CrossRef]
18. Okada, H., Masujin, K., Iwamaru, Y., Imamura, M., Matsuura, Y., Mohri, S., Czub, S. and Yokoyama, T. 2011. Experimental transmission of h-type bovine spongiform encephalopathy to bovinized transgenic mice. *Vet. Pathol.* **48**: 942–947. [Medline] [CrossRef]
19. Okada, H., Iwamaru, Y., Imamura, M., Masujin, K., Matsuura, Y., Shimizu, Y., Kasai, K., Mohri, S., Yokoyama, T. and Czub, S. 2011. Experimental H-type bovine spongiform encephalopathy characterized by plaques and glial- and stellate-type prion protein deposits. *Vet. Res.* **42**: 79. [Medline] [CrossRef]
20. Parchi, P., Chen, S. G., Brown, P., Zou, W., Capellari, S., Budka, H., Hainfellner, J., Reyes, P. F., Golden, G. T., Hauw, J. J., Gajdusek, D. C. and Gambetti, P. 1998. Different patterns of truncated prion protein fragments correlate with distinct phenotypes in P102L Gerstmann-Sträussler-Scheinker disease. *Proc. Natl. Acad. Sci. U.S.A.* **95**: 8322–8327. [Medline] [CrossRef]
21. Piccardo, P., Dlouhy, S. R., Lievens, P. M., Young, K., Bird, T. D., Nochlin, D., Dickson, D. W., Vinters, H. V., Zimmerman, T. R., Mackenzie, I. R., Kish, S. J., Ang, L. C., De Carli, C., Pocchiari, M., Brown, P., Gibbs, C. J. Jr., Gajdusek, D. C., Bugiani, O., Ironside, J., Tagliavini, F. and Ghetti, B. 1998. Phenotypic variability of Gerstmann-Sträussler-Scheinker disease is associated with prion protein heterogeneity. *J. Neuropathol. Exp. Neurol.* **57**: 979–988. [Medline] [CrossRef]
22. Prusiner, S. B. 1991. Molecular biology of prion diseases. *Science* **252**: 1515–1522. [Medline] [CrossRef]
23. Satoh, K., Muramoto, T., Tanaka, T., Kitamoto, N., Ironside, J. W., Nagashima, K., Yamada, M., Sato, T., Mohri, S. and Kitamoto, T. 2003. Association of an 11-12 kDa protease-resistant prion protein fragment with subtypes of dura graft-associated Creutzfeldt-Jakob disease and other prion diseases. *J. Gen. Virol.* **84**: 2885–2893. [Medline] [CrossRef]
24. Tagliavini, F., Prelli, F., Porro, M., Rossi, G., Giaccone, G., Farlow, M. R., Dlouhy, S. R., Ghetti, B., Bugiani, O. and Frangione, B. 1994. Amyloid fibrils in Gerstmann-Sträussler-Scheinker disease (Indiana and Swedish kindreds) express only PrP peptides encoded by the mutant allele. *Cell* **79**: 695–703. [Medline] [CrossRef]
25. Zou, W. Q., Capellari, S., Parchi, P., Sy, M. S., Gambetti, P. and Chen, S. G. 2003. Identification of novel proteinase K-resistant C-terminal fragments of PrP in Creutzfeldt-Jakob disease. *J. Biol. Chem.* **278**: 40429–40436. [Medline] [CrossRef]