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## RESEARCH ARTICLE




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# Evaluation of proteinase K-resistant prion protein (PrP<sup>res</sup>) in Korean native black goats carrying a potential scrapie-susceptible haplotype of the prion protein gene (*PRNP*)

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## ABSTRACT

Prion disease is a fatal neurodegenerative disease with a broad host range in humans and animals. It is caused by proteinase K-resistant prion protein (PrP<sup>res</sup>). In previous studies, a heterogeneous infection in Cervidae and Caprinae was reported. Chronic wasting disease (CWD) has been frequently reported as the only prion disease in Korea that occurs in livestock. Thus, there is a possibility of transmission of CWD to Korean native black goats. However, PrP<sup>res</sup> has not been investigated thus far in Korean native black goats. We found strong linkage disequilibrium between c.126G>A and c.414T>C ( $r^2 = 1$ ) and between c.718C>T and c.126G>A ( $r^2 = 0.638$ ). In addition, the haplotype GTGTAAAC (representing codons 42, 102, 127, 138, 143, 146, 218 and 240) showed the highest frequency with 45.1%. Among 41 Korean native black goats, 20 animals (48.78%) were homozygous for the susceptible haplotypes (histidine at codon 143, asparagine at codon 146 and arginine at codon 154). Interestingly, we did not detect PrP<sup>res</sup> bands in any of the tested animals, including the 20 animals carrying potential scrapie susceptible haplotypes.

## KEYWORDS

prion, prion disease, polymorphisms, scrapie, goat, PrP<sup>res</sup>, *PRNP*

## INTRODUCTION

Scrapie is a transmissible spongiform encephalopathy (TSE) that occurs in sheep and goats. It is a neurodegenerative disease characterised by abnormal behaviour, lip-smacking, blind-folding, altered gaits and dysmetria (Greenlee, 2019; Kim et al., 2019a, 2019b; Cassmann and Greenlee, 2020). TSE includes chronic wasting disease (CWD) in elk and deer, bovine spongiform encephalopathy (BSE) in cattle, feline spongiform encephalopathy (FSE) in cheetahs, pumas and cats, and Creutzfeldt-Jakob disease (CJD) in humans (Imran and Mahmood, 2011; Jeong and Kim, 2014; Benestad and Telling, 2018; Kim and Jeong, 2018; Houston and Andreoletti, 2019; Won et al., 2020).

BSE is the only zoonotic prion disease that can spread from cattle to humans (Prusiner, 1991; Brandel and Knight, 2018; Houston and Andreoletti, 2018). However, a previous study revealed that scrapie is directly transmitted to cynomolgus macaques with an extended incubation period after intracerebral inoculation (Comoy et al., 2015). This result suggests the possibility of scrapie transmission to primates. In addition, recent studies have reported that scrapie and CWD agents from mule deer are transmitted to white-tailed deer and sheep,

respectively, via intracerebral inoculation (Hamir et al., 2006; Greenlee et al., 2011). Scrapie was reported in sheep and goats in several European, American and Asian countries (Hunter et al., 1997; Thorgeirsdottir et al., 1999; Colussi et al., 2008; Bouzalas et al., 2010; Papasavva-Stylianou et al., 2011; Ortiz-Pelaez et al., 2015; Srithayakumar et al., 2016; Vouraki et al., 2018; Matsuura et al., 2019). In Korea, only CWD has been reported in the elk population (Sohn et al., 2002; Kim et al., 2005; Lee et al., 2013). Taken together, these results suggest that there is a possibility of transmission of CWD to goats. Thus, the pre-emptive examination of scrapie is very important in goats, which are major hosts of scrapie.

Previous studies have reported that genetic variants of the caprine prion protein gene (*PRNP*), including I142M, N146S, R211Q and Q222K, are associated with susceptibility to scrapie in goats (Billinis et al., 2002; Corbiere et al., 2013). Korean native goats with the caprine *PRNP* gene showed a high occurrence of the haplotype susceptible to scrapie infection (Kim et al., 2019a). Until now, screening tests for scrapie have not been conducted in Korean native black goats.

In the present study, we performed scrapie diagnosis in the medulla oblongata of 41 Korean native black goats using Western blot analysis and analysed the haplotype distribution of the *PRNP* gene.

## MATERIALS AND METHODS

### Ethical statement

All experimental procedures were reviewed and approved by the Institute of Animal Care and Use Committee (IACUC) of Jeonbuk National University (CBNU 2018-037, CBNU 2017-0076).

### Samples and scrapie strain

The ME7 scrapie strain was provided by The Roslin Institute at the University of Edinburgh. The brain samples of 41 Korean native black goats were collected at slaughterhouses in the Republic of Korea.

### Genetic analysis

Genomic DNA was extracted using a Hi-Yield Genomic DNA Mini Kit (Real Biotech Corporation, Taipei, Taiwan). Polymerase chain reaction (PCR) was performed to amplify the goat *PRNP* gene using goat *PRNP* gene-specific primers. Primer information is as follows: forward primer (5'-ATTTTGCAGAGAAGTCATCATGGTGA-3') and reverse primer (5'-TAGGATAGGGGCAACCTTCCTGTT-3'). These primers were designed based on the goat *PRNP* gene listed on the GenBank website (Gene ID: 102169975) and amplified the open reading frame (ORF) of the goat *PRNP* gene. A 25-μL mixture containing 2.5 μL of 10X *Taq* DNA polymerase buffer, 1 μL of genomic DNA (40–50 ng/μL), 10 pmol each primer, 0.5 μL of a 0.2 μM dNTP mixture, 5 μL of

5X Band Helper, 0.25 μL of *Taq* DNA polymerase and 15.75 μL of sterile deionised water was used for PCR. The PCR conditions were as follows: denaturing at 95 °C for 2 min, 34 cycles of 95 °C for 20 s, annealing at 58 °C for 40 s, extension at 72 °C for 1 min 30 s, and a final extension at 72 °C for 5 min. The PCR products were obtained by the FavorPrep GEL/PCR Purification Mini Kit (FAVORGEN, Taiwan). The purified PCR products were directly sequenced with an ABI 3730 sequencer (ABI, Foster City, California, USA), and the sequencing results were analysed using Finch TV software (Geospiza Inc., Seattle, USA).

### Statistical analysis

Haplotype analysis and linkage disequilibrium (LD) tests were used to calculate the  $r^2$  value and were performed using Haploview version 4.2 (Broad Institute, Cambridge, MA, USA).

### Western blot analysis

A total of 41 samples of medulla oblongata were homogenised in 10% volumes of RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, USA) containing complete protease inhibitor cocktail (Roche, Germany). The brain homogenates were centrifuged at 14,000 rpm for 15 min at 4 °C. The protein concentration of the supernatant was measured by bicinchoninic acid (BCA) assay (BioRad, USA). To investigate the PrP<sup>res</sup> band, 1 μL of proteinase K (20 μg/mL) was treated to 80 μg of the supernatant at 37 °C for 15 min. Equal amounts of protein (80 μg) were heated at 95 °C for 15 min. Subsequently, the samples were loaded on a 12% sodium dodecyl sulphate (SDS) gel with a loading buffer containing 0.125 M Tris-HCl (pH 6.8), 20% glycerol, 5% SDS, 10% mercaptoethanol and 0.002% bromophenol blue. The separated proteins were transferred to nitrocellulose membranes (Amersham, USA) using an electrophoretic transfer system (BioRad, USA) at 100 V for 1 h. The membranes were washed with a Tris-buffered saline solution (pH 7.6) containing 0.05% Tween 20 (TBST), and nonspecific binding was blocked by TBST containing 5% skim milk for 2 h at room temperature. The membrane was then incubated at 4 °C for 24 h with mouse monoclonal anti-PrP antibody (SAF32, 1:200). After washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody against mouse immunoglobulin G (IgG) (Sigma-Aldrich, USA) for 1 h at room temperature and washed in TBST again. The target signals were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, USA).

## RESULTS

### LD analysis of the *PRNP* polymorphisms in Korean native black goats

We investigated the genotype and allele frequencies of polymorphisms of the *PRNP* gene in 41 Korean native black

Table 1. Linkage disequilibrium (LD) analysis of *PRNP* polymorphisms in Korean native black goats

	c.126G>A 42P	c.304T>G W102G	c.379G>A G127S	c.414T>C 138S	c.428A>G H143R	c.437A>G N146S	c.652A>C I218L	c.718C>T P240S
c.126G>A 42P	–							
c.304T>G W102G	0.077	–						
c.379G>A G127S	0.038	0	–					
c.414T>C 138S	1.0	0.077	0.038	–				
c.428A>G H143R	0.097	0.008	0.004	0.097	–			
c.437A>G N146S	0.03	0.044	0.001	0.03	0.028	–		
c.652A>C I218L	0.038	0	0	0.038	0.004	0.001	–	
c.718C>T P240S	0.638	0.121	0.06	0.638	0.062	0.019	0.06	–

goats and found a total of 8 single nucleotide polymorphisms (SNPs). To investigate whether there was a strong LD among the 8 *PRNP* polymorphisms, namely, c.126G>A (42P), c.304T>G (W102G), c.379G>A (G127S), c.414T>C (138S), c.428A>G (H143R), c.437A>G (N146S), c.652A>C (I218L) and c.718C>T (P240S), we performed LD analysis with the  $r^2$  value (Table 1). A strong LD ( $r^2 = 1$ ) was identified between c.126G>A and c.414T>C. In addition, c.718C>T showed a strong LD ( $r^2 = 0.638$ ) with c.126G>A and c.414T>C.

### Haplotype analysis of the *PRNP* polymorphisms in Korean native black goats

Among the eight haplotypes, the haplotype GTGTAAAC showed the highest frequency with 45.1%; the second major haplotype was GTGTGAAC with 23.2% for Korean native black goats (Table 2). In previous studies, the histidine allele at codon 143, the asparagine allele at codon 146 and arginine at codon 154 were found to be related to susceptibility to scrapie (Vaccari et al., 2009). Thus, we investigated the haplotype frequency of codons 143, 146 and 154 and annotated the susceptibility in Korean native black goats

(Table 3). Notably, 20 out of 41 Korean native black goats (48.78%) were homozygous for the susceptible haplotype (HNR/HNR).

### A test for scrapie in the medulla oblongata of Korean native black goats using Western blot analysis

The PrP<sup>res</sup> control band was examined in brain homogenates of healthy mice (Fig. 1, lanes 1–2) and ME7 scrapie-infected mice (Fig. 1, lanes 3–4). In a healthy mouse, the PrP<sup>res</sup> band was not detected in the brain homogenate treated with proteinase K (Fig. 1, lane 2), whereas in a ME7 scrapie-infected mouse the PrP<sup>res</sup> band was detected in the brain homogenate treated with proteinase K (Fig. 1, lane 4). Subsequently we investigated the PrP<sup>res</sup> band in the medulla oblongata samples of 41 Korean native black goats, including the 20 Korean native black goats carrying a potential scrapie-susceptible haplotype (HNR/HNR), and a representative image is shown in Fig. 1 (lanes 5–8). The PrP<sup>res</sup> band was not detected in any of the 41 Korean native black goats, including the 20 Korean native black goats carrying potential scrapie-susceptible haplotypes (Fig. 1, lanes 6 and 8; Table 3).

Table 2. Haplotype analysis of *PRNP* polymorphisms in Korean native black goats

	c.126G>A 42P	c.304T>G W102G	c.379G>A G127S	c.414T>C 138S	c.428A>G H143R	c.437A>G N146S	c.652A>C I218L	c.718C>T P240S	Observed number (frequency)
Haplotype 1	G	T	G	T	A	A	A	C	37 (0.451)
Haplotype 2	G	T	G	T	G	A	A	C	19 (0.232)
Haplotype 3	A	T	G	C	A	A	A	T	11 (0.134)
Haplotype 4	G	T	G	T	A	G	A	C	6 (0.073)
Haplotype 5	A	T	G	C	A	A	A	C	5 (0.061)
Haplotype 6	A	G	G	C	A	A	A	T	2 (0.025)
Haplotype 7	A	T	G	C	A	A	C	C	1 (0.012)
Haplotype 8	A	T	A	C	A	A	A	T	1 (0.012)

Table 3. Detailed information on the potential scrapie susceptibility-related *PRNP* haplotype and results of the PrP<sup>res</sup> inspection

	Haplotype (codons 143, 146, 154)	Susceptibility	PrP <sup>res</sup>
Goat 1	HNR/HNR	Sus/Sus	Negative
Goat 2	HSR/HSR	NA/NA	Negative
Goat 3	HNR/HNR	Sus/Sus	Negative
Goat 4	HNR/RNR	Sus/NA	Negative
Goat 5	HNR/RNR	Sus/NA	Negative
Goat 6	RNR/RNR	NA/NA	Negative
Goat 7	HNR/RNR	Sus/NA	Negative
Goat 8	HNR/HSR	Sus/NA	Negative
Goat 9	HNR/HSR	Sus/NA	Negative
Goat 10	RNR/RNR	NA/NA	Negative
Goat 11	HNR/HNR	Sus/Sus	Negative
Goat 12	HNR/HNR	Sus/Sus	Negative
Goat 13	HNR/HNR	Sus/Sus	Negative
Goat 14	HNR/HNR	Sus/Sus	Negative
Goat 15	HNR/RNR	Sus/NA	Negative
Goat 16	RNR/RNR	NA/NA	Negative
Goat 17	HNR/HNR	Sus/Sus	Negative
Goat 18	HNR/HNR	Sus/Sus	Negative
Goat 19	HNR/RNR	Sus/NA	Negative
Goat 20	HNR/HNR	Sus/Sus	Negative
Goat 21	HNR/HNR	Sus/Sus	Negative
Goat 22	HNR/HNR	Sus/Sus	Negative
Goat 23	HNR/HNR	Sus/Sus	Negative
Goat 24	HNR/RNR	Sus/NA	Negative
Goat 25	HNR/RNR	Sus/NA	Negative
Goat 26	HNR/RNR	Sus/NA	Negative
Goat 27	HNR/LNR	Sus/NA	Negative
Goat 28	RNR/RNR	NA/NA	Negative
Goat 29	HNR/RNR	Sus/NA	Negative
Goat 30	HNR/HSR	Sus/NA	Negative
Goat 31	HNR/HNR	Sus/Sus	Negative
Goat 32	HNR/HNR	Sus/Sus	Negative
Goat 33	HNR/HSR	Sus/NA	Negative
Goat 34	HNR/HNR	Sus/Sus	Negative
Goat 35	HNR/RNR	Sus/NA	Negative
Goat 36	HNR/HNR	Sus/Sus	Negative
Goat 37	HNR/HSR	Sus/NA	Negative
Goat 38	HNR/HNR	Sus/Sus	Negative
Goat 39	HNR/HNR	Sus/Sus	Negative
Goat 40	HNR/HNR	Sus/Sus	Negative
Goat 41	HNR/HNR	Sus/Sus	Negative

Shaded boxes indicate Korean native black goats carrying potential susceptible haplotypes (HNR) in homozygous form. Sus: susceptible; NA: not applicable

## DISCUSSION

Previous studies have reported that SNPs at codons 143, 146, 154, 211 and 222 of the caprine *PRNP* gene are associated with scrapie susceptibility (Vaccari et al., 2009; Fragkiadaki et al., 2011; White et al., 2012; Ricci et al., 2017). In our previous study (Kim et al., 2019a), we found a total of three major haplotypes, HNR/RQ (62.9%), RNR/RQ (30.3%) and HSR/RQ (4.5%), in 211 Korean native black goats which were predicted to have a low aggregation propensity by AMYCO (<http://bioinf.uab.es/amyco04/>). Since a confirmatory test of PrP<sup>res</sup> in Korean native black goats has not been

performed thus far, we performed a PrP<sup>res</sup> test through Western blot analysis in medulla oblongata samples from 41 Korean native black goats (Fig. 1). In the present study, we did not find SNPs at *PRNP* codons 211 and 222 in any of the tested animals. Thus, we analysed the susceptible haplotype at *PRNP* codons 143, 146 and 154 (Table 3). Notably, more than 40% of Korean native black goats were homozygous for the susceptible haplotype. In addition, we performed a screening test for scrapie and did not observe a PrP<sup>res</sup> band in any of the 41 Korean native black goats, including the animals carrying a potential scrapie-susceptible haplotype of the *PRNP* gene.



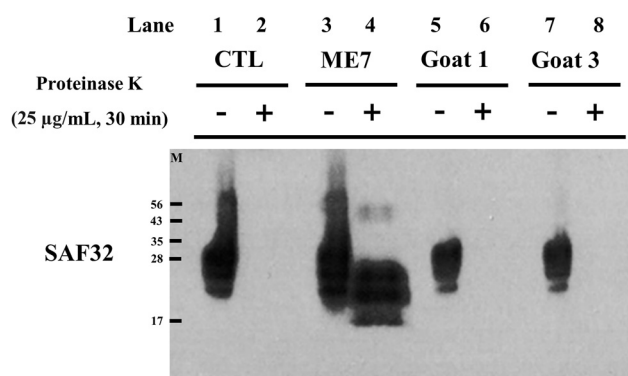


Fig. 1. Detection of PrP<sup>res</sup> in Korean native black goats by Western blotting. Lane 1: Proteinase K-untreated whole brain from healthy C57BL/6 mice. Lane 2: Proteinase K-treated whole brain from healthy C57BL/6 mice. Lane 3: Proteinase K-untreated whole brain from C57BL/6 mice inoculated with ME7. Lane 4: Proteinase K-treated whole brain from C57BL/6 mice inoculated with ME7. Lane 5: Proteinase K-untreated medulla oblongata from Korean native black goat 1. Lane 6: Proteinase K-treated medulla oblongata from Korean native black goat 1. Lane 7: Proteinase K-untreated medulla oblongata from Korean native black goat 2. Lane 8: Proteinase K-treated medulla oblongata from Korean native black goat 2. Bars on the left indicate the molecular size markers (in kilodaltons). CTL: negative control, healthy C57BL/6 mice; ME7: positive control, C57BL/6 mice inoculated with ME7 strain; Goat 1: Korean native black goat 1; Goat 3: Korean native black goat 3; -: samples not treated with proteinase K; +: proteinase K-treated samples

According to a previous study (Kim et al., 2019a), the major haplotypes of Korean native black goats are predicted to have low aggregation propensity, which is likely to contribute to the absence of scrapie-infected cases in Korean native black goats. However, as the average slaughtering age of Korean black goats used in this study is less than 2 years and the average onset age of scrapie is 3–4 years (Baylis and Goldmann, 2004), this slaughtering age may not be sufficient to detect scrapie-related PrP<sup>res</sup>. It is highly desirable to conduct scrapie diagnosis in larger samples of Korean native black goats using Western blot analysis, immunohistochemistry, protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QUIC) in the future (Barletta et al., 2005; Saa et al., 2006).

Recent studies have reported that the soil is contaminated with PrP<sup>res</sup> through the faeces. Since CWD was spreading through horizontal transmission on several farms in Korea (Sohn et al., 2002; Lee et al., 2013), PrP<sup>res</sup>-contaminated soil may be a prominent causative agent of CWD (Kuznetsova et al., 2018; Sohn et al., 2019). That is why it is necessary to continuously monitor Korean native black goats, which have the potential for CWD cross-contamination.

In conclusion, we found 8 haplotypes of the *PRNP* gene in Korean native black goats. In addition, we screened Korean native black goats for PrP<sup>res</sup>. All tested animals, including those with the potential scrapie susceptible-haplotype of the *PRNP* gene, were negative for PrP<sup>res</sup>. To the best of our knowledge, this was the first study to use a PrP<sup>res</sup>

detection test in goats carrying a susceptible haplotype of the *PRNP* gene.

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