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NO POLYMORPHISMS IN THE CODING REGION OF THE PRION-LIKE PROTEIN GENE IN THOROUGHBRED RACEHORSES

Min-Ju JEONG[1,2] and Byung-Hoon JEONG[1,2*]

[1]Korea Zoonosis Research Institute, Chonbuk National University, 820-120 Hana-ro, Iksan, Jeonbuk 54531, Republic of Korea; [2]Department of Bioactive Material Sciences, Chonbuk National University, Jeonju, Jeonbuk, Republic of Korea

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Prion diseases are fatal neurodegenerative diseases characterised by the accumulation of an abnormal prion protein isoform (PrP^{Sc}), which is converted from the normal prion protein (PrP^{C}). Prion diseases have been reported in an extensive number of species but not in horses up to now; therefore, horses are known to be a species resistant to prion diseases. The prion-like protein gene (*PRND*) is closely located downstream of the prion protein gene (*PRNP*) and the prion-like protein (Doppel) is a homologue with PrP. Previous studies have shown that an association between prion diseases and polymorphisms of the *PRND* gene is reported in the main hosts of prion diseases. Hence, we examined the genetic variations of the *PRND* gene in Thoroughbred horses. Interestingly, polymorphisms of the *PRND* gene were not detected. In addition, we conducted a comparative analysis of the amino acid sequences of the *PRND* gene to identify the differences between horses and other species. The amino acid sequence of the horse *PRND* gene showed the highest identity to that of sheep (83.7%), followed by that of goats, cattle and humans. To the best of our knowledge, this is the first genetic study of the *PRND* gene in horses.

Key words: Horses, prion, *PRND*, polymorphisms, Thoroughbred, resistance

Prion diseases are rare, but they are lethal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in deer (Prusiner, 1998; Williams, 2005; Goldmann, 2008; Jeong and Kim, 2014; Curcio et al., 2016). Although there is a wide spectrum of hosts involved in prion diseases, there have been no reports of prion diseases occurring in horses to date.

*Corresponding author; E-mail: bhjeong@jbnu.ac.kr; Phone: 0082 (63) 900-4040; Fax: 0082 (63) 900-4012

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Horses as well as dogs and chickens are known to be resistant to prion diseases (Perez et al., 2010; Moore et al., 2011; Zhang, 2011; Zhang et al., 2013; Qing et al., 2014; Kim et al., 2018), even though horses have a long lifespan of approximately 30 years (Perez et al., 2010). Previous studies have tried to find a specific factor to bestow the resistance of prion diseases in horses, and many researchers have focused on the structure of the prion protein (PrP) and its stability in horses because prion diseases are caused by the accumulation of abnormal prion protein (PrP^{Sc}), the misfolded form of normal prion protein (PrP^{C}). In addition, the polymorphisms of the prion protein gene (*PRNP*), which encodes PrP, could affect the structure and stability of PrP and are known to be associated with susceptibility to prion diseases (Jeong et al., 2005*c, d*; Kurt et al., 2014).

The prion-like protein gene (*PRND*) is located 20 kb downstream of the *PRNP* gene, and the prion-like protein (Doppel) shares significant biochemical and structural homology with PrP (Moore et al., 1999; Jeong et al., 2005*a*). The distribution of polymorphism at the 3' untranslated region (UTR) +28 site of the human *PRND* gene has shown significant differences between sporadic CJD and healthy control subjects (Croes et al., 2004; Jeong et al., 2005*a, b*). In cattle, statistical analysis of BSE-affected and healthy animals indicated that the genotype distributions of codons 95 and 132 in the bovine *PRND* gene are significantly different in Fleckvieh breeds (Balbus et al., 2005). In addition, recently it has been reported that the genotype of codon 26 of the ovine *PRND* gene is significantly linked to the disease-associated haplotype of the ovine *PRNP* gene and affects sperm capacitation and fertilisation ability in rams (Pereira et al., 2009; Mesquita et al., 2010). In horses, polymorphisms of the *PRNP* gene have been reported (Kim and Jeong, 2018*b*); however, polymorphisms of candidate genes related to prion disease have not been investigated thus far.

In this study, we investigated single nucleotide polymorphisms (SNPs) within the open reading frame (ORF) of the *PRND* gene in 242 Thoroughbred horses. In addition, we compared the amino acid sequences of the *PRND* gene between horses and several other species.

Materials and methods

Ethical statement

All blood samples of 242 Thoroughbred horses were provided by the Seoul Race Park in South Korea. All experimental procedures were approved by the Chonbuk National University Institutional Animal Care and Use Committee (Korea) (CBNU 2016-65).

Genetic analysis of the PRND *gene*

The DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate genomic DNA from 200 µl of peripheral whole blood following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using the following gene-specific forward and reverse primers: PRND-F (5'-GCCCGT TGCAGCTTCTTATCT-3') and PRND-R (5'-GCTGGAGGAGAGAAGTGG GAT-3'). The PCR mixture comprised 2.5 µl of 10× Taq DNA polymerase buffer, 0.5 µl of 10 mM dNTP mixture, 1 µl each of forward and reverse primers, 2.5 µl of 5× Band Helper, 0.2 µl of Taq DNA polymerase (Promega, Fitchburg, WI, USA), 1 µl of genomic DNA and sterile water to reach the final volume of 25 µl. PCR was conducted in an S-1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA), and the PCR cycling parameters were as follows: 95 °C for 2 min, followed by 32 cycles of 95 °C for 20 sec, 61 °C for 40 sec, 72 °C for 1 min, and then 1 cycle of 72 °C for 5 min for the final extension. Amplified PCR products were purified by means of the Gel Extraction Kit (Qiagen) and were sequenced with an ABI PRISM 3730XL Analyzer (ABI, Foster City, CA, USA). The sequencing result was decoded using Finch TV software (Geospiza Inc., Seattle, WA, USA) and genotyping was performed.

Comparison of the amino acid sequence of the PRND *gene*

Sequence alignment was performed using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalo/). The amino acid sequences of the *PRND* gene, including humans (*Homo sapiens*, NC_000020.11), cattle (*Bos taurus*, AC_000170.1), sheep (*Ovis aries*, NC_019470.2), and goats (*Capra hircus*, NC_030820.1), except for horses (*Equus caballus*, in this study), were acquired from GenBank at the National Center for Biotechnology Information (NCBI) website.

Results

We amplified the 868 bp targeting the ORF region of the *PRND* gene in 242 Thoroughbred horses and performed automatic direct sequencing on the fragments. Interestingly, no polymorphisms were detected in the ORF region of the horse *PRND* gene (Fig. 1).

In addition, we compared the results from horses with the distributions of the *PRND* polymorphisms reported previously in humans, sheep, goats, and cattle. At least two or more polymorphisms in the ORF of the *PRND* gene were reported in several animal species except horses (Table 1). Next, we performed multiple sequence alignments among various species using ClustalW2. The amino acid sequence of the *PRND* gene in horses was similar to that in sheep with the highest homology (83.7%), followed by that in goats (83.1%), cattle (82.6%) and humans (75.3%) (Fig. 2).

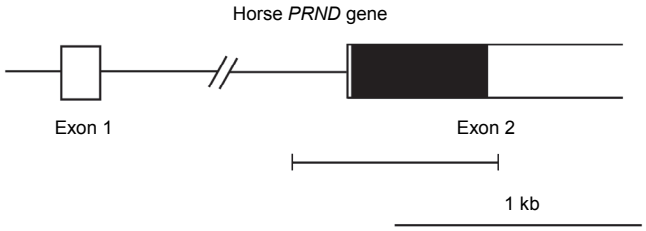

Fig. 1. Genomic map of the prion-like protein gene (*PRND*) in horses. Schematic diagram denotes the genomic structure of the horse *PRND* gene, drawn to scale. The open reading frame (ORF) within exon 2 is indicated by the black block, and white blocks indicate the 5' and 3' untranslated regions (UTRs). The edged horizontal bar indicates the regions sequenced. No polymorphisms of the *PRND* gene in Thoroughbred horses were found in this study

Table 1

Distribution of polymorphisms at the open reading frame (ORF) of the prion-like protein gene (*PRND*) in various species

Species	Polymorphisms	Total, n	Reference
Humans	p.Thr26Met (T26M) p.Pro56Leu (P56L) p.Thr174Met (T174M)	3	Peoc'h et al., 2000; Jeong et al., 2005*b*
Cattle	p.Arg50His (R50H) p.Ser95Ser (S95S) p.Asn110His (N110H) p.Arg132Gln (R132Q) p.Ile176Ile (I176I)	5	Comincini et al., 2001; Balbus et al., 2005; Kim and Jeong, 2018*a*
Sheep	p.Ile12Ile (I12I) p.Ala26Ala (A26A)	2	Comincini et al., 2001; Pereira et al., 2009
Goats	p.Leu10Leu (L10L) p.Ser22Phe (S22F) p.Ile33Ile (I33I) p.Thr51Ala (T51A) p.Val129Leu (V129L) p.Glu96Lys (E96K)	6	Uboldi et al., 2005; Jeong et al., 2018
Horses	ND*	0	This study

*ND: not detected

Discussion

In previous studies, many researchers have reported that salt bridges and the β2–α2 loop in the horse *PRNP* gene particularly provide structural stability so that horses can exhibit low susceptibility to prion disease (Perez et al., 2010; Zhang, 2011; Kurt et al., 2014). They revealed that salt bridges of the horse PrP

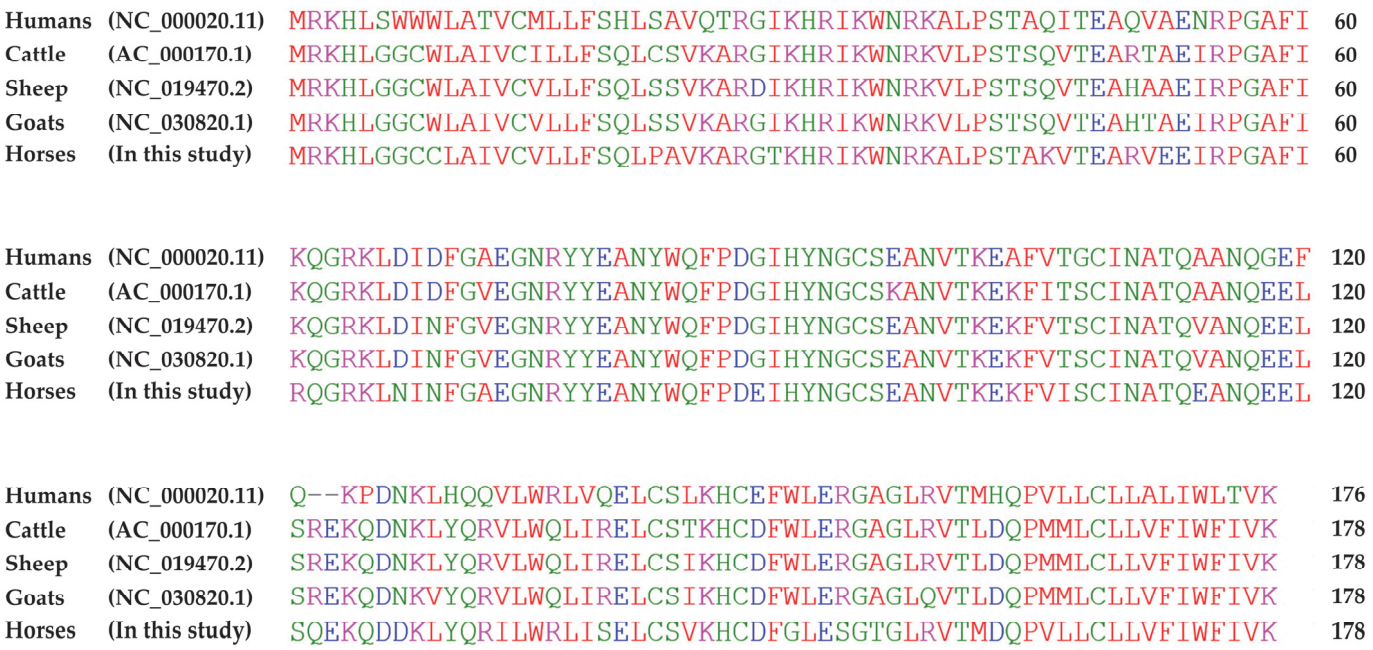

Fig. 2. Comparison of the amino acid sequences of the *PRND* gene in humans, cattle, sheep, goats, and horses. The amino acid sequences were aligned by ClustalW2. The amino acid sequences of the *PRND* gene include the following: humans, NC_000020.11; cattle, AC_000170.1; sheep, NC_019470.2; goats, NC_030820.1; Thoroughbred horses, this study. Colours indicate the chemical properties of amino acids: blue: acidic; red: small and hydrophobic; magenta: basic; green: hydroxyl, sulphhydryl, amine, and glycine

can contribute to the maintenance of a stable molecular structure in a disordered environment by linking the structure of β2-sheet and α2-helix (Zhang, 2011). In addition, an amino acid alteration of the *PRNP* codon 167 from aspartic acid, which was conserved in various mammals, to serine, was only identified in horses and resulted in a well-defined structure of the β2–α2 loop, consequently presenting a noteworthy stability of the horse PrP (Perez et al., 2010; Sanchez-Garcia and Fernandez-Funez, 2018). Nevertheless, it is still proposed that further work should be conducted in horses.

To identify the disease-related polymorphisms in various species, we tried to study the prion protein gene family, including *PRNP, PRND* and prion-related protein (*PRNT*) genes (Jeong et al., 2005*d*; Jeong et al., 2013; Kim and Jeong, 2017*a,b*; Jeong et al., 2018; Kim and Jeong, 2018*a,b,c*; Kim et al., 2018; Won et al., 2019). In previous studies, we found only two insertion and deletion polymorphisms in the chicken *PRNP* gene and one SNP in the horse *PRNP* gene (Kim and Jeong, 2018*b*; Kim et al., 2018). In this study, polymorphisms were not found in the ORF of the horse *PRND* gene (Fig. 1). Considering that the amino acid sequence of the *PRND* gene in horses was quite conserved in comparison with that in other species (Fig. 2), the absence of polymorphisms in the *PRND* gene is an unusual finding, which is first confirmed in Thoroughbred horses (Table 1). Therefore, it is necessary to investigate the genetic characteristics of the *PRND* gene in horse breeds other than the Thoroughbred in the future.

Among the various breeds of horses, Thoroughbred horses are known to be a preferable and valuable breed in the horse racing industry and have been selectively bred to nurture the outstanding qualities of racehorses (Lee et al., 2014). It is unclear whether the polymorphism of the horse *PRND* gene was not found in this study due to inbreeding for the preservation of the Thoroughbred lineage. However, many polymorphisms of the candidate genes related to race performance have been reported in Thoroughbred horses (Gu et al., 2010; Hill et al., 2010). In addition, the registered Thoroughbred racehorses in Seoul Race Park originated from over 10 pedigrees and 9 countries including New Zealand, the USA, Ireland, England, Japan, Canada, France, Australia, and Korea. Furthermore, the major portion of racehorses in Seoul Race Park has a low proximity coefficient of inbreeding (~0–0.2). It is implied that a variable genetic background exists in Thoroughbreds registered in Seoul Race Park. Since the total number of Thoroughbred racehorses raised in Korea is 16,854, the randomly selected 242 Thoroughbred samples in this study can represent the Thoroughbred population raised in Korea. In a previous study, a lot of SNPs in Thoroughbred samples obtained from Seoul Race Park have been identified using next-generation sequencing (Moon et al., 2015). Thus, it is estimated that the absence of *PRND* SNPs in Thoroughbred horses was not due to inbred status or small sample size; rather, it is a notable genetic feature of the horse, an animal resistant to prion disease. In this regard, further studies are needed to determine whether

the rare occurrence of polymorphisms in the *PRND* and *PRNP* genes has a significant effect on prion disease susceptibility.

In summary, we investigated the polymorphisms of the *PRND* gene in 242 Thoroughbred horses and carried out a comparative analysis of the *PRND* gene between horses and several other species. The amino acid sequences of the *PRND* gene were closely similar between the diverse species; however, polymorphisms in the ORF of the horse *PRND* gene were not detected. To the best of our knowledge, this study is the first genetic report of the *PRND* gene in horses.

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