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RESEARCH PAPER

The first report of genetic variations in the chicken prion protein gene

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ABSTRACT

Abnormal structural changes of the prion protein (PrP) are the cause of prion disease in a wide range of mammals. However, spontaneous infected cases have not been reported in chicken. Genetic variations of the prion protein gene (*PRNP*) may impact susceptibility to prion disease but have not been investigated thus far. Because an investigation of the chicken *PRNP* can improve the understanding of characteristics related to resistance to prion disease, research on the chicken *PRNP* is highly desirable. In this study, we investigated the genetic characteristics of the chicken *PRNP* gene. For this, we performed direct sequencing in 106 Dekalb White chickens and analyzed the genotype and allele frequencies of chicken *PRNP* gene. We found two insertion and deletion polymorphisms in the chicken *PRNP*: c.163_180delAACCCAGGGTACCCCCAT and c.268_269insC. The former is a U2 hexapeptide deletion polymorphism. Of the 106 samples, 13 (12.26%) were insertion homozygotes, 89 (83.96%) were heterozygotes, and 4 (3.77%) were deletion homozygotes in c.163_180delAACCCAGGGTACCCCCAT. In the c.268_269insC polymorphism, 102 (96.23%) were deletion homozygotes, and 4 (3.77%) were heterozygotes. Insertion homozygotes of c.268_269insC were not detected. Two polymorphisms were in perfect linkage disequilibrium (LD) with a D' value of 1.0, and three haplotypes were identified. Furthermore, PROVEAN evaluates 163_180delAACCCAGGGTACCCCCAT as 'deleterious' with a score of – 13.173. Furthermore, single nucleotide polymorphisms (SNPs) in the open reading frame (ORF) of the *PRNP* gene were not found in the chicken. To the best of our knowledge, this was the first report on the genetic variations of the chicken *PRNP* gene.

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Introduction

The prion protein (PrP) is coded by the prion protein gene (*PRNP*), and aberrant structural changes from the cellular form (PrP^{C}) to the scrapie form of the prion protein (PrP^{Sc}) causes prion diseases in mammals[1]. Previous studies have tried to identify the cause of prion disease; however, the apparent cause of onset is elusive. To date, spontaneous prion disease in birds has not been reported, and parenteral and oral challenge of prion agent has failed to infected chickens [2,3]. Thus, several studies have attempted to find the feature of the chicken prion protein that leads to prion disease resistance.

In previous studies, it has been reported that homology of the prion protein was very low between mammals and birds; however, important domains, such as signal peptide, tandem repeat domain (octapeptide in mammals and hexapeptide in birds), hydrophobic region and C-terminal globular domain, are conserved[4]. Although amino acids compositions of tandem repeat are different, octapeptide and hexapeptide showed identical copper related functions in mammals and chickens, respectively[5]. In addition, the expression level of mRNA and protein of *PRNP* was similar in the central nervous system of both species [6–8]. This finding indicates that the major function and expression profile of the prion protein are identical, although several differences existed between mammals and birds. In a recent study, molecular dynamics studies found that the prion protein of chicken has an stable molecular structure analogous to that of humans, which is considered as a susceptible species to prion disease [9]. Because a highly stable molecular structure of the prion protein has been reported in horses, dogs and rabbits, which are classified as resistant animals to prion disease [10–13], there may be another factor that contributes to prion disease resistance in chickens.

The genetic variations of the *PRNP* gene that can potently impact prion disease susceptibility have not been investigated in chicken. The genetic variations of the *PRNP* gene are significantly related to prion disease susceptibility in a wide range of hosts and are subdivided into three groups according to the type and location. The first group is non-synonymous single nucleotide polymorphisms

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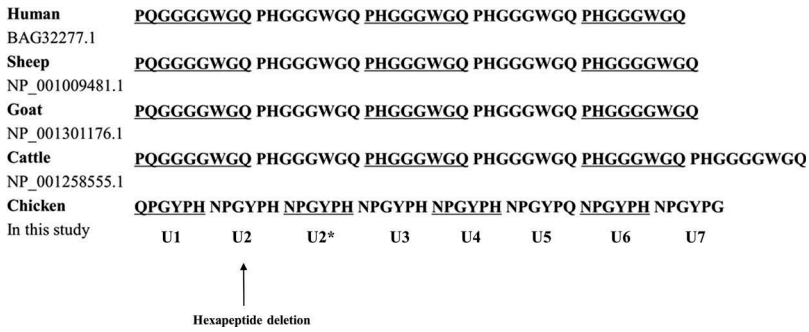

Figure 1. Comparisons of tandem repeat sequence in humans, sheep, goat, cattle and chicken. Tandem repeat sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI), including those of human (*Homo sapiens*, BAG32277.1), sheep (*Ovis aries*, NP_001009481.1), goat (*Capra hircus*, NP_001301176.1), cattle (*Bos taurus*, NP_001258555.1) and chicken (*Gallus gallus*, in this study). Arrows indicate the hexapeptide deletion polymorphism found in this study. Hexapeptide repeat units were named from U1 to U7 in turn. Word spacing and underlines discriminate consecutive tandem repeat units.

(SNPs) and mutations in the C-terminal globular domain of PrP^C. SNPs are the most commonly observed form and are detected at codons 129, 180, 200, and 219 in humans [14,15], codons 136, 154, and 171 in sheep [16,17], codons 142, 143, 146, 154, 171, 211, and 222 in goat [18,19], and codons 95 and 96 in deer [20,21]. The second group is insertion/deletion polymorphisms in the promoter region of the *PRNP* gene. The number of prion disease-affected cattle was correlated with 23-bp insertion/deletion in the promoter region [22]. The third group is octapeptide insertion/deletion polymorphism in the N-terminal octapeptide domain. Octapeptide insertion is significantly associated with human prion disease onset [23]. Because investigation of genetic characteristics of chicken *PRNP* gene may be important in understanding the functions of PrP^C, which is a pre-disease substrate of prion disease, a trial to identify genetic features of chicken *PRNP* gene is meaningful.

The purpose of this study was to investigate genetic characteristics of the chicken *PRNP* gene. To do so, we performed direct sequencing in 106 Dekalb White chickens and investigated genotype and allele frequencies of the chicken *PRNP* gene. In addition, we evaluated the impact of polymorphisms on chicken prion protein using PROVEAN.

Results

To investigate the genotype and allele frequencies of chicken *PRNP* gene polymorphism, we performed PCR to amplify the chicken *PRNP* gene. Chicken *PRNP* amplicons were automatically and directly sequenced in 106 Dekalb White chickens using ABI 3730 DNA sequencer. Because tandem repeats of chicken are significantly different from those of mammals, we aligned tandem repeats among mammals and chickens (Figure 1). Interestingly, we identified only insertion and deletion polymorphisms in the ORF of the chicken *PRNP* gene. A total two insertion and deletion polymorphisms were identified: c.163_180delAACCAGGGTACCCCCAT and c.268_269insC (Figure 2). c.163_180delAACCCAGGG TACCCCCAT is a hexapeptide deletion polymorphism. To designate the location of the hexapeptide deletion polymorphisms, we named the tandem repeat from U1 to U7. The identified hexapeptide deletion is located on U2 (Figure 1). These are the first electropherograms showing a deletion of a single hexapeptide repeat in chicken using an automatic DNA sequencer (Figure 3). Another polymorphism is c.268_269insC. Interestingly, c.268_269insC entails homozygosity of U2 deletion (data not shown). In addition, the insertion homozygote of c.268_269insC was not detected in this study. Electropherograms of c.268_269insC was first reported in this study (Figure 4). Furthermore, to validate two *PRNP* alleles and investigate the haplotype between two polymorphisms in the sample #44, which has heterozygous genotypes at two polymorphisms, the PCR products of chicken *PRNP* gene from the sample #44 was amplified, subcloned, and sequenced. Only two alleles were observed in sample #44 with WT-WT alleles (clone 1) and deletion-insertion alleles (clone 2) (Figure 5). Detailed genotype and allele frequencies of these polymorphisms are described in Table 1.

To examine if there was a strong linkage disequilibrium (LD) among the 2 polymorphisms, LD (|D'|) was calculated for the chicken *PRNP* gene. All 2 polymorphisms of chicken *PRNP* gene were in perfect LD with D' values 1.0 (Table 2). Analysis of the haplotype frequency was carried out in Dekalb White. As shown in Table 3, three different haplotypes exist in the chicken *PRNP* polymorphisms. Among the three haplotypes, the most frequent haplotype is ht1 (54.24%), followed by ht2 (43.87%) and ht3 (1.89%) (Table 3).

Next, to evaluate the impact of insertion/deletion polymorphisms on chicken PrP^C, we utilized PROVEAN.

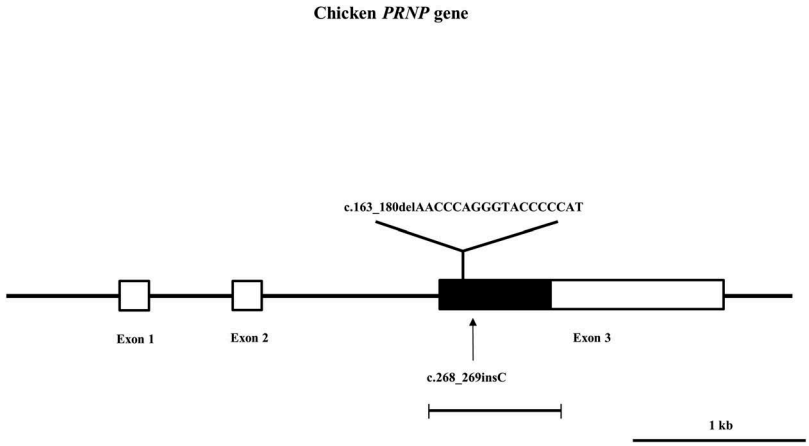

Figure 2. Gene map and polymorphism identified in the chicken prion protein gene (*PRNP*) on chromosome 22. The open reading frame (ORF) is indicated by a shaded block and the 5′ and 3′ untranslated regions (UTRs) are indicated by white blocks. Horizontal bars with edges indicate the regions sequenced. Arrows indicate the polymorphisms found in this study. The Y-shaped bar indicates the hexapeptide deletion polymorphisms identified in the chicken *PRNP* gene.

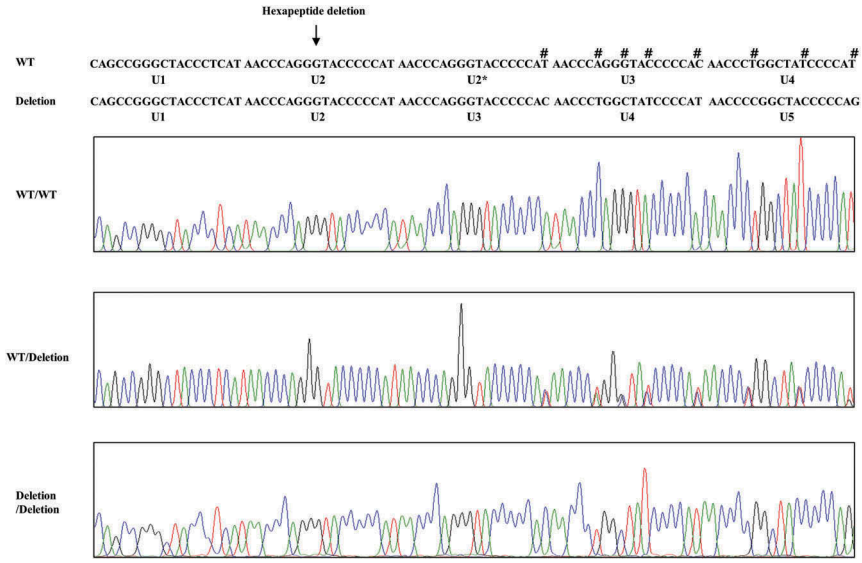

Figure 3. Electropherogram of hexapeptide deletion polymorphism in chicken *PRNP* gene. Four colors indicate individual bases of DNA sequence using ABI 3730 automatic sequencer (blue: cytosine, red: thymine, black: guanine, green: adenine). Sharps (#) indicate double peaks induced by hexapeptide deletion polymorphism. Arrows indicate the hexapeptide deletion polymorphism (U2) found in this study. WT: wild-type of chicken hexapeptide repeat. Deletion: U2 deletion polymorphisms of chicken hexapeptide repeat.

PROVEAN can measure the impact of insertion/deletion protein polymorphisms. Surprisingly, PROVEAN estimated hexapeptide deletion as 'Deleterious' with a score of −13.173 (Table 4).

Discussion

In previous studies, because *PRNP* knockout mice showed resistance to prion agents and relatively normal physiology, the function of PrP^C was unclear [24,25]. However, Brown *et al* found that PrP^C (23–98 residues) has copper binding capacity and *PRNP* knockout mice showed significantly lower copper concentrations in the brain among several divalent cations[26]. In addition, these *PRNP* knockout mice showed reduced copper dependent enzyme reactivity. Thus, these results indicate that PrP^C has copper binding ability. Indeed, synthetic octapeptide repeat, which is located on N-terminal domain of the prion protein, confirmed the copper binding domain of PrP^C[27]. Among the tandem repeat regions, 'PHGGGWGQ' is an octapeptide repeat unit. Specifically, the imidazole ring of histidine is significantly related to the interaction between PrP^C and

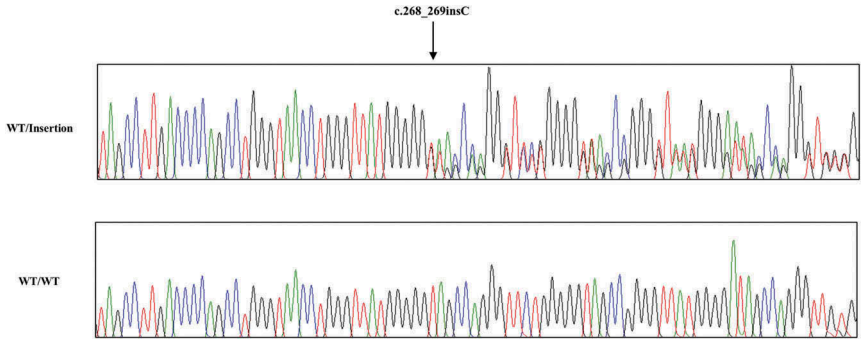

Figure 4. Electropherogram of c.268_269insC polymorphism in chicken *PRNP* gene. Four colors indicate individual bases of DNA sequence using ABI 3730 automatic sequencer (blue: cytosine, red: thymine, black: guanine, green: adenine). Sanger sequencing was performed by reverse gene-specific primer, as described in the materials and method section. Arrows indicate c.268_269insC polymorphism found in this study. Upper portion: heterozygote of c.268_269insC polymorphism. Lower portion: wild-type of the chicken *PRNP* gene sequence. WT: wild-type of the chicken *PRNP* gene sequence. Insertion: c.268_269insC polymorphisms of the chicken *PRNP* gene sequence.

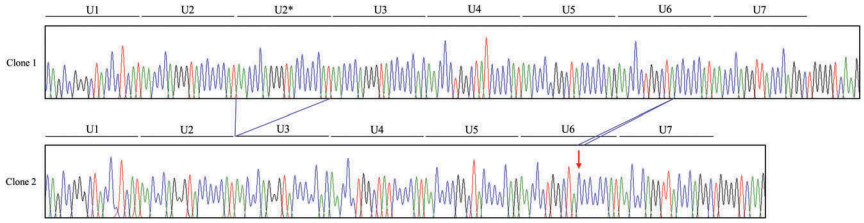

Figure 5. Electropherogram of the chicken *PRNP* region encompassing hexapeptide deletion and c.268_269insC polymorphisms in sample #44. Clone 1: Electropherogram showed wild-type sequences in hexapeptide deletion and c.268_269insC polymorphisms from the sample #44. Clone 2: The deletion sequence in hexapeptide deletion polymorphism and the insertion sequence at c.268_269insC polymorphism from the sample #44. Arrow indicates c.268_269insC polymorphism found in this study.

Table 1. Genotype and allele frequencies of the *PRNP* polymorphisms in chickens.

		Genotype frequency, n (%)			Allele frequency	
Polymorphisms	Total, n	+/+	+/−*	−/−	+	−
c.163_180delAACCCAGGGTACCCCCAT	106	13	89	4	115	97
c.268_269insC	106	0	4	102	4	208

*+ indicates an insertion; − indicates a deletion

Table 2. Linkage Disequilibrium (LD) among two polymorphisms of *PRNP* in chickens.

	c.163_180delAACCCAGGGTACCCCCAT	c.268_269insC
c.163_180delAACCCAGGGTACCCCCAT	–	1.0
c.268_269insC	–	–

Table 3. Haplotype frequency of two *PRNP* polymorphisms in chickens.

Haplotype	c.163_180delAACCCAGGGTACCCCCAT	c.268_269insC	n (%)
ht1	WT	WT	115 (54.24)
ht2	DEL	WT	93 (43.87)
ht3	DEL	INS	4 (1.89)

copper [28,29]. In a recent study, neuroprotective α-cleavage event of PrP^C significantly influences on Cu (II) coordination at its His111 site [30].

However, this well conserved octapeptide repeat sequence was found only in mammals. Chicken has a significantly different form of tandem repeat, called hexapeptide repeat. Due to this significantly different composition of amino acids of the tandem repeat, we performed sequence alignment and compared sequence differences of tandem repeat domains (Figure 1). In previous studies, the repeat unit of chicken hexapeptide repeat is controversial [31,32]. Here, we suggested a

Table 4. Prediction of protein functional alteration induced by deletion polymorphism of the chicken prion protein using PROVEAN.

Polymorphism	Score	Prediction
c.163_180delAACCCAGGGTACCCCCAT	−13.173	Deleterious

novel tandem repeat unit based on polymorphisms of the chicken *PRNP* gene: U1 to U7 (Figure 1). Interestingly, heterozygosity of hexapeptide deletion genotype frequencies was found in 89 (83.96%) of 106 Dekalb White chickens. Because octapeptide in/del polymorphisms in mammals are rare, highly polymorphic hexapeptide polymorphism in chicken *PRNP* gene are noticeable. Thus, the heterozygosity of the hexapeptide deletion genotype does not significantly affect survival of chicken. In fact, in a previous study, it was reported that two amino acids, histidine and tyrosine, provide copper binding ability of the chicken prion protein. Since chicken has twice the number of amino acids in the tandem repeat unit compared mammals (mammals: histidine, chicken: histidine, tyrosine), it was presumed that the impact of a single deletion of hexapeptide seems to be relatively low and does not act as selection pressure [33]. In addition, we did not find SNPs in the chicken *PRNP* gene. Since the *PRNP* gene is highly polymorphic in mammals and several disease-associated SNPs were identified [34–37], it is noteworthy that no SNP was found in the chicken *PRNP* gene. Since the chicken samples have been collected from an animal farm, it is possible that whole genetic diversity of chicken isn't reflected in this study. Thus, future studies will be necessary to identify SNPs of the chicken *PRNP* gene in large groups and other breeds of the chicken.

Lastly, we assessed the impact of hexapeptide deletion polymorphism on the protein. For this, we used PROVEAN to evaluate the impact of c.163_180delAACCCAGGGTACCCCCAT.
PROVEAN measured this deletion protein polymorphism as 'Deleterious' with a score of −13.173. This score indicates that hexapeptide deletion may make impact prion protein function. In a recent *in vitro* functional study, because it was reported that the number of hexapeptide repeat units is directly proportional to copper binding ability [32], the heterozygotes or homozygotes of U2 deletion found in this study demonstrated decreased copper binding ability. An exact estimate of copper binding ability according to hexapeptide genotype is highly desirable in the future.

Overall, our study suggested three main points. First, we investigated chicken *PRNP* gene polymorphisms and found only in/del polymorphisms. Second, we suggested a novel tandem repeat unit of chicken hexapeptide repeat based on polymorphisms. Third, we evaluated the impact of deletion of hexapeptide polymorphisms *in silico*. To the best of our knowledge, this was the first report of chicken *PRNP* gene polymorphisms.

Materials and methods

Ethics statement

A total of 106 Dekalb White (3 weeks old) chickens were obtained from an animal farm in South Korea. All experimental processes performed in the present study approved according to regulations of the Animal Care and Use Committee of Chonbuk National University (IACUC Number: CBNU 2017–0030).

DNA extraction

Genomic DNA was isolated from 20 mg peripheral tissue using the LaboPass Tissue Genomic DNA Isolation Kit (Cosmo Genetech CO., Ltd., Korea) following the manufacturer's instructions.

Genetic analysis

Polymerase chain reaction (PCR) was carried out with gene-specific sense and antisense primers as follows: chicken PRNP-F (TGGGATGATGCTTGATTTCGGT) and chicken PRNP-R (ATCCCTGTCACGCTCCAGAA) using S-1000 Thermal Cycler (Bio-Rad Laboratories, USA). The PCR reagents contained 25 pmol of each primer, 5 µl of 10 × *Taq* DNA polymerase buffer, 1 µl of 10 mM dNTPs and 2.5 units of *Taq* DNA polymerase (Promega, USA). The PCR conditions were as follows: 94°C for 2 min to denature, and 35 cycles of 94°C for 45 sec, 63°C for 45 sec, 72°C for 1 min 30 sec, and then 1 cycle of 72°C for 10 min to extend the reaction. A 5 µl aliquot of the PCR product was analyzed by electrophoresis on a 1.2% agarose gel stained with EcoDye™ DNA Staining Solution (BIOFACT, KOREA) to confirm the target band size (978 bp). The PCR product (sample # 44) was subcloned into pGEM-T easy vector (Promega, USA). The recombinant plasmid pGEM-T easy vector was transformed into the *E. coli* TOP 10 competent cells (Thermo Fisher Scientific, USA). The purified PCR products and the positive clones were performed with Sanger DNA sequencing using ABI 3730 automatic sequencer with *Taq* Dideoxy Terminator Cycle Sequencing Kit (ABI, USA). The sequencing result was processed by Finch TV software (Geospiza Inc, Seattle, USA), and genotyping of the chicken *PRNP* gene was performed. The haplotypes of two polymorphisms in the chicken *PRNP* gene were

analyzed by SNP Analyzer 1.2A (http://snp.istech21.com/snpanalyzer/1.2A/). We also examined Lewontin's D' (|D'|) to investigate LD among these two polymorphisms by SNP Analyzer 1.2A.

Evaluation impact of insertion/deletion to chicken PrP^{C}

PROVEAN assesses the impact of polymorphisms by building up and comparing the clusters of related sequences and predicting the score. If the final score is below −2.5, protein variants are predicted to be 'neutral'; if the final score is above −2.5, protein variants are predicted as 'deleterious' (http://provean.jcvi.org/seq_submit.php).

Abbreviations

LD	linkage disequilibrium
ORF	open reading frame
PRNP	prion protein gene
PrP	prion protein
PrP^{C}	cellular form of prion protein
PrP^{Sc}	scrapie form of the prion protein
SNP	single nucleotide polymorphism
UTRs	untranslated regions

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Disclosure statement

No potential conflict of interest was reported by the authors.

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