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First Identification of the Causal Mutation for Coagulation F11 Deficiency in Hanwoo Cattle

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Plasma thromboplastin antecedent (factor XI or F11) deficiency is a relatively mild hemorrhagic genetic disorder reported in Holstein and Japanese black cattle that is caused by two insertional mutations. In this study, we identified a causal mutation of F11 deficiency in Hanwoo cattle and developed a more efficient method to identify this mutation. A total of 2,043 Hanwoo samples from 35 farms, representing three regions (Yeongam, Haman, Yanggu) were used in this study. The mutation is caused by the insertion of 15 bp at exon 9 of the F11 gene; the same mutation has been described in Japanese black cattle. In addition, the gene frequency of F11 deficiency was 1.52% in the Hanwoo cattle population, and all mutant alleles appeared in the heterozygous animals. Especially, this result suggests that 15 bp insertion might be caused by a semi–embryonic lethal gene, which could affect the Hanwoo population. Although, the proportion of the disorder allele is low, the system for verifying this deficiency and additional phenotypic symptoms are needed to prevent any kind of losses in Hanwoo industry. Also the novel TaqMan assay genotyping method for the identification of F11 deficiency proposed in this study offers rapid and accurate results and may be efficiently applied in Hanwoo breeding programs.

Key words: Factor XI, Hanwoo, Genetic disorder, TaqMan genotyping

INTRODUCTION

Plasma thromboplastin antecedent (factor XI or F11) is a plasma serine proteolytic coenzyme involved in the early stage of blood coagulation reactions. The cattle F11 gene is located on bovine chromosome 27 (BTA27) and consists of 15 exons and 14 introns. The structure of F11 is composed of two types of homodimers (heavy chain and light chain) linked by a disulfide bond (Bouma and Griffin, 1977). The heavy chain homodimers comprise four Apple domains, which have binding sites for F9, F12, thrombin, and high–weight kininogen (Baglia *et al*., 1990, 1993; Baglia and Walsh, 1996; Sun and Gailani, 1996). Of these, F11 is converted into an activated form by F12, which performs a series of roles to activate F9.

F11 deficiency has been reported in several mammals including humans, dogs, and cattle, which inherit it in the recessive autosomal form (Dodds and Kull, 1971; Gentry *et al*., 1975; Kociba *et al*., 1969). Other than hemophilia, the symptoms of F11 deficiency are relatively mild. Rare consequences of F11 deficiency include natural hemorrhage and joint bleeding. In cattle, the causative mutation of F11 deficiency can be classified into two types. F11 deficiency reported in Japanese black cattle (Type I) is caused by the insertion of 15 bp in the exon 9 region and a single nucleotide polymorphism (SNP) located at the end of the insertion site in the F11 gene, which is converted from adenine to cytosine in the amino acid sequence (Takasu *et al*., 2005). A mutation identified in Holstein cattle (Type II) is caused by a stop codon resulting from a 76–bp insertion in the F11 gene at exon 12 (Marron *et al*., 2004).

Although some studies have been conducted on genetic diseases in Hanwoo cattle, genetic disease monitoring systems have not yet been established (Chung *et al*., 2005). Therefore, relatively mild genetic diseases, such as F11 deficiency, cannot be identified in Hanwoo cattle. In addition, Hanwoo breeding systems have been extensively used across Korea to select proven bulls through progeny tests and artificial insemination. Thus, it is necessary to identify carrier animals, even when they have normal phenotypes, to ensure the removal of defective alleles from the entire Hanwoo population. Therefore, the purpose of this study was to identify F11 deficiency in Hanwoo cattle and to determine the status of F11 deficiency on farms.

MATERIALS AND METHODS

1. Animals and preparation of genomic DNA samples

Animals was made up at the Hanwoo Farm in Yeongam County (10 farms; 776 animals), in Haman County (15 farms; 822 animals) and in Yanggu (10 farms; 445 animals). Total 2,043 of blood samples were used for this experiment.

Blood samples prepared for DNA extraction was centrifuged at a speed of 300 g to separate the leukocyte

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layer located in the buffy coat. DNA extraction was performed on the separated leukocyte layer using two methods. First, gDNA was extracted using the PrimePrep ™ genomic DNA isolation kit (Genetbio, Korea) as a method for extracting genomic DNA (gDNA) using a general DNA extraction kit. The concentration and quality of the extracted DNA were measured using a Nanodrop 2000 c spectrophotometer (Thermo Scientific, USA). DNA samples that underwent QC procedures were subjected to 25 ng / μL dilution for the same PCR amplification. Second, gDNA was extracted using PrimePrep Direct PCR Reagent (Genetbio, Korea) to simplify the DNA extraction process and to test the rapid and easy genotyping process. In this method, 5 μl of leukocyte layer and 100 μl of Reagent solution were mixed at a ratio of 1:20, and the reaction time was 10 minutes at 95˚C. After that, the supernatant was obtained by centrifugation and diluted 20 times to perform Taq Man assay genotyping.

2. PCR amplification and genotyping

We designed and used three pairs of primers specific to the mutation site of the F11 gene to identify the cause mutation of the F11 deficiency in Hanwoo (Table 1) and PCR (polymerase chain reaction) amplification was used to confirm the genotype.

A genotyping was carried out through electrophoresis to check indels of PCR amplification product. F11 deficiency mutations proceeded only by simple electrophoresis without additional treatment and restriction enzyme as insertion mutation.

3. Sequencing and TaqMan assay genotyping

PCR sequence data was obtained by direct sequencing (www.cosmogentech.co.kr) of the PCR products that were purified from 10 normal animals and 3 carrier animals. The obtained sequence information was analyzed using BioEdit Sequence Alignment Editor program (BioEdit, USA).

TaqMan assay genotyping was performed by specific probe assay that was designed for identify F11 indel region in Hanwoo. Genotyping was performed by using a Bio–Rad real–time PCR machine (Bio–Rad, USA) to isolate mutant and normal individuals as fluorescence colors of VIC and FAM, respectively.

RESULTS AND DISCUSSION

To identify the causative mutation of F11 deficiency, 376 Hanwoo samples were initially subjected to polymerase chain reaction (PCR) amplification and electrophoresis using a specific primer set that identified Type I and Type Ⅱ mutations. The Type Ⅱ deficiency mutation was not detected in the Hanwoo population. However, normal animals with the Type I mutation showed only one PCR fragment (342 bp), whereas carrier animals showed two different PCR fragments (179 and 342 bp) resulting from a 15–bp insertional mutation (Figure 1). To confirm these results, we performed direct sequencing of the carrier animals and found that the results matched those of mutant alleles in Japanese black cattle. Lim *et al*. (Lim *et al*., 2016) previously investigated Type Ⅱ F11 deficiency in 78 proven Hanwoo bulls and found no mutations, consistent with the results of the current study. Kim *et al*. (Kim *et al*., 2002) examined the genetic distances among Hanwoo, Holstein, and Japanese black cattle using 13 highly polymorphic microsatellite markers and determined that Hanwoo and Japanese black cattle were clustered together, whereas the Holstein breed clustered separately. Similarly, a principal components analysis (PCA) using 58 SNP makers to assess the genetic diversity of cattle revealed that Japanese black cattle were genetically more closely related to Hanwoo than Holstein cattle (Lin *et al*., 2010). The Type I mutation identified in the current study may support the close relationships found between Hanwoo and Japanese black cattle in these previous studies.

F11 deficiency in Holstein cattle has been reported to prolong the reproductive cycle due to the retardation of luteal atrophy caused by weakness in follicular development during the estrous cycle (Liptrap *et al*., 1995), and has been associated with low fertility in animals

Table 1. Primer pair information for F11 indel identification in two kinds of exon area and Taq Man probe information for F11 mutation identification in Hanwoo

Marker	Gene	Location	Region	Primer (Forward/Reverse) (5'–3')	Amplicon (bp)	annealing temp (˚C)
Primer1	Coagulation factor 11 gene	g.15367049–124–(76bp insert)	Exon12	AGCGGCATTTTGAATCAATC/ ATGGACTGAAAGGGGAGCTT	287	53
Primer2		g.15362363–77–(15bp insert)	Exon9	ACTTTCAAAATGGGAACTCCTTCC/ ATGGCAGAACACTGCACAGC	342	64
Primer3			Exon9	TGCTGTGCAGTGTTATATGTGC/ TTGCACGATTCTTGAGATGG	179	64

Taq Man Assay probe	Coagulation factor 11 gene	g.15362363–77–(15bp insert)	Exon9	Primer (Forward/Reverse) (5'–3')	Probe (Wild/Mutant) (5'–3')	Fluorescent color (Wild/Mutant)
				GTTTTGCCTTTCACATCTCAATATGT/ GAAATCAGTGTTGCGATAGAATGAAG	CTGTGCAGTGTTCTG/ ATATGTGCAGAATATATGCC	FAM / VIC

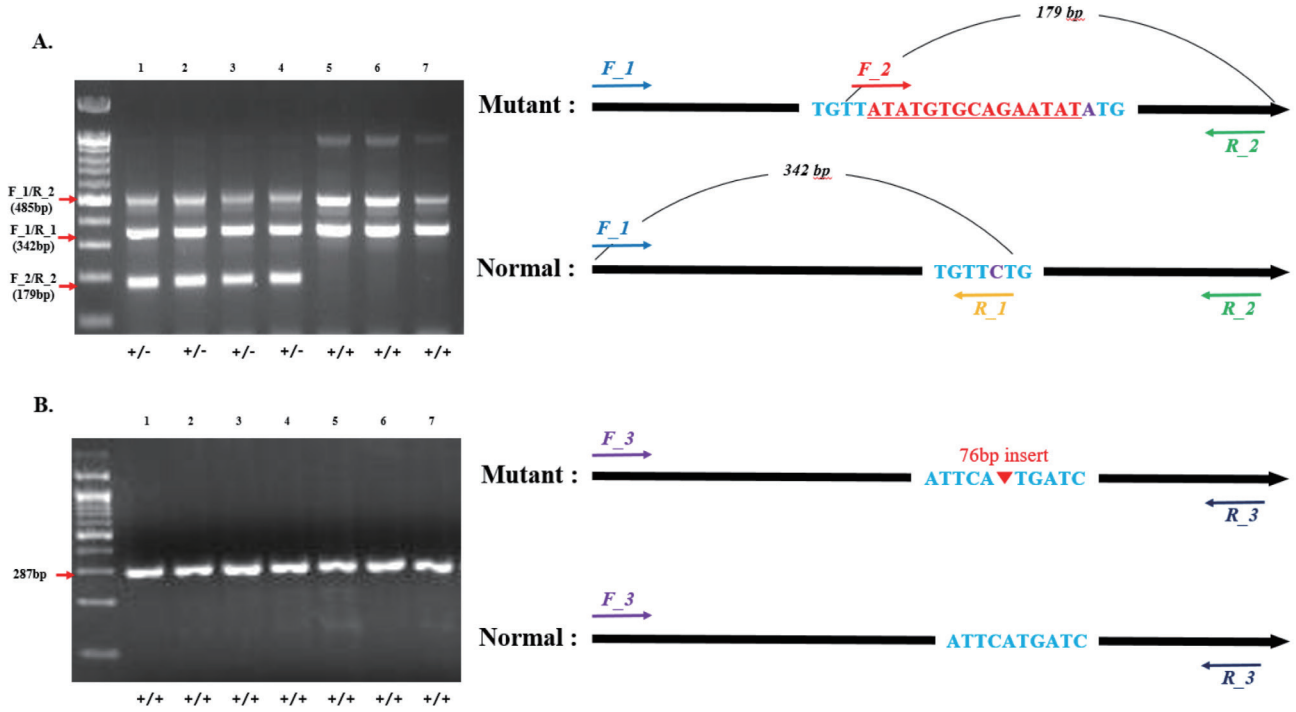

Fig. 1. The electrophoresis results for two types of F11 deficiency. (A) The carrier animals (+/−) having type I mutant showed both 485 bp and 179 bp fragments, that resulted by 15 bp insertional mutation. The normal animals (+/+) showed both 485 bp and 342 bp fragments. (B) Only normal animals, showing 287 bp, for the type II deficiency were identified in this study.

affected by F11 deficiency (Ghanem *et al.*, 2005). Holsteins with low–fertility symptoms due to F11 deficiency were calculated to have caused a total financial loss of more than $300 per affected cow compared to normal cows (Akyuz *et al.*, 2012).

Symptoms of F11 deficiency in Japanese black cattle tend to be mild, and there have been no reports of bleeding symptoms except for exudative hemorrhage due to castration or dehorning. Although no reports of abnormal reproductive traits have been published, F11 deficiency might lead to symptoms of low fertility; a study of Holstein cattle showed an increase in calving interval of about 50 days (Akyuz *et al.*, 2012). In contrast, Japanese black calves with F11 deficiency exhibited growth retardation and pelvic dysplasia. These results suggest that F11 deficiency can impact calf growth and development (Takasu *et al.*, 2005); similar symptoms can be expected in Hanwoo cattle. However, no reports to date have indicated the need for further phenotype measurement and genetic relationship studies in Hanwoo cattle to validate observations of F11 deficiency symptoms in this species. The pedigree information of the carrier animals has been traced to proven Hanwoo bulls (KPN) 872, 879, and 919, suggesting that the allele causing this disorder is in both proven bulls and cows from individual farms. Therefore, further investigation of F11 deficiency symptoms in KPN and cows is needed. In particular, we could not detect recessive homozygous animals in the population examined in this study, suggesting that F11 deficiency might be caused by a semi–embryonic lethal gene, which could affect the Hanwoo population. It is necessary to accurately measure the Hanwoo cattle F11 deficiency phenotype and its effects on productivity. In addition, the risk of declining productivity in Hanwoo cattle should be eliminated through testing for F11 deficiency both in commercial stocks and in proven bulls (KPN).

The activated partial thromboplastin (APPT) test is a conventional method used to identify F11 deficiency by measuring F11 activity (Gentry *et al.*, 1975). Although the causes of F11 deficiency in Holstein and Japanese black cattle are different, the range of F11 activity between normal and carrier animals overlaps in both varieties (Kunieda *et al.*, 2005; Liptrap *et al.*, 1995). Therefore, the diagnosis of F11 deficiency through APPT makes it difficult to determine the carrier animals; this situation is also expected to affect F11 diagnosis in the Hanwoo cattle population. Furthermore, although PCR amplification is a basic and intuitive method to identify F11 deficiency, it requires approximately 6 hours. Therefore, we performed TaqMan assay genotyping as a new method for accurate identification of carrier and affected animals with F11 deficiency.

The results with Hanwoo cattle showed that 1,981 of 2,043 animals (96.97%) were identified as normal, and 62 (3.03%) as heterozygous animals for the Type I F11 deficiency. In contrast, no F11 deficiency–affected animals (recessive homozygous) were identified. Thus, the gene frequency for F11 deficiency was 1.51% (Table 2).

We also compared the genotype results of various conditions to overcome difficulties in identifying genotypes (i.e., carrier and normal in the current study) that may occur in the field. There were two main objectives

in designing this study. The first was to confirm whether the minimum genomic DNA concentration required for genotyping is identified by clear separation (Figure 2C). DNA was were diluted 10, 20, 30, 40, 50, and 60 times with 5 ng/μL of DNA at the initial concentration for efficient testing of the method. The results were the same for all dilution conditions: clearly separated genotypes between normal (orange circle) and carrier animals (green triangle) (Figure 2). Furthermore, these results confirmed that F11 deficiency can be identified using a 60× dilution of 5 ng gDNA (roughly 83.3 pg). The second objective was to reduce the time required for F11 deficiency genotyping. In this experiment, we used the PCR reagent to skip the routine DNA extraction step, reducing the time required for DNA extraction. As a result, the total amount of time required to identify F11 deficiency from the blood sample was only about 2 hours (Figure 2B). The TaqMan assay genotyping method for F11 deficiency identification was able to clearly detect both affected and carrier animals and also significantly reduced the time and the total amount of genomic DNA required for mutant allele detection.

This study is the first report of the presence of a Type I mutation for F11 deficiency in Hanwoo cattle, and we propose TaqMan assay genotyping as an efficient method for the identification of F11 deficiency. Our results can be used to improve the efficiency of Hanwoo cattle breeding programs in the future.

AUTHOR CONTRIBUTIONS

Sung Hyun CHO, Performed experiment, data analysis, manuscript writing.

Dongwon SEO, Ph. D; Experimental design, manuscript writing.

Onolragchaa GANBOLD, Performed experiment,

Table 2. Number of Hanwoo animals identified in each genotype for the F11 deficiency in three geographical locations in Korea

Location in Korea	Total No of Animal	Number of animals in each Genotype			mutant allele frequency (%)
		Normal	Carrier	Mutant	
Yeongam	776	752	24	0	1.5
Haman	822	796	26	0	1.6
Yanggu	445	433	12	0	1.3
Total	2,043	1,981	62	0	1.5

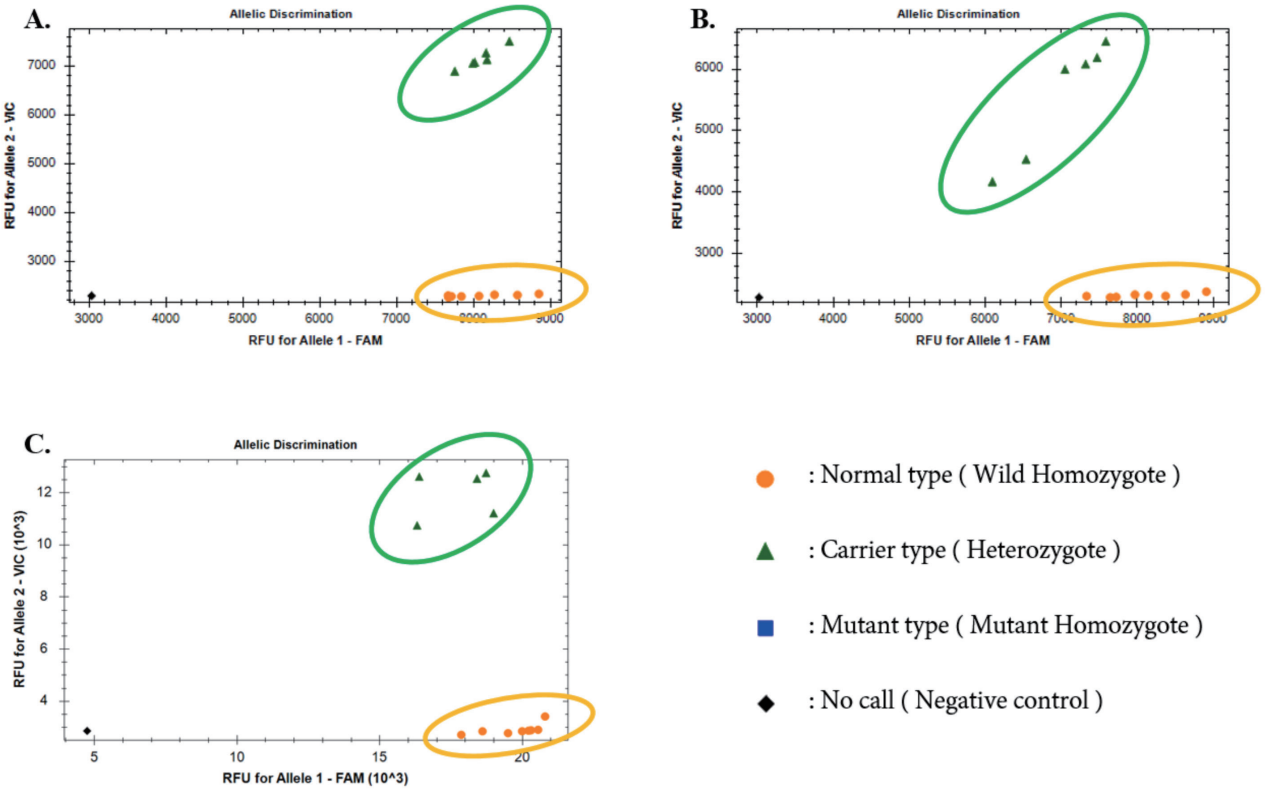

Fig. 2. The results of three different experiments using the F11 Taq Man assay showed the same results. (A) 5 ng diluted DNA. (B) The results using Direct PCR Reagent for saving experiment time. (C) Experiment using roughly 83.3 pg DNA indicates the same results compared with diluted 5 ng of DNA concentration.

data analysis.

Nu Ri CHOI, Ph. D; Performed experiment.

Prabuddha MANJULA, Performed experiment.

Shil JIN, Ph. D; Performed experiment.

Seung Hwan LEE, Ph. D; Manuscript editing.

Nobuhiko YAMAUCHI, Ph. D; Manuscript editing.

Takafumi GOTOH, Ph. D; Manuscript editing.

Jun Heon LEE, Ph. D; Experimental design, manuscript editing.

AUTHOR DISCLOSURE STATEMENT

None of the authors have any competing financial interests to declare.

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