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Full Length Article

Investigation of NELFE Polymorphism and their Association with Litter Size in Two Chinese Indigenous Sheep Breeds

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Abstract

Litter size is an important index for assessing productivity and economic benefits in the sheep industry. The NELFE gene, which is associated with embryonic development and mammalian reproduction, was studied as a candidate gene to identify molecular markers associated with Small-tailed Han and Hu sheep prolificacy. In total, 275 Hu sheep and 318 Small-tail Han sheep were included in this study, and single nucleotide polymorphism of the ovine NELFE gene were detected in the two highly prolific sheep breeds by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and DNA sequencing. Consequently, two polymorphism were identified in intron 1 (g.454T>C) and intron 2 (g.883A>C). Within the g.883A>C locus, two genotypes (AA and AC) were found, and minor allele frequency was smaller than 0.01 (MAF=0.003), which was not subject to do association study. Three genotypes (TT, TC, and CC) were detected in the g.454T>C locus. Of these, TC was the dominant genotype with a frequency of 0.62, and C allele was the dominant allele with a frequency of 0.52. Association analysis of g.454T>C with litter size revealed that ewes with the TC genotype had 0.25 ($P < 0.05$) and 0.15 ($P > 0.05$) more lambs than ewes with TT and CC genotypes, respectively. Furthermore, Hu ewes with TC genotype had 0.39 ($P < 0.05$) and 0.27 ($P < 0.05$) more lambs than those with TT and CC genotypes, while this significant association was not observed in Small-tail Han sheep ($P > 0.05$). Based on our results, marker-assisted selection using NELFE is potentially warranted to increase litter size in sheep and may be of considerable economic value to sheep producers. © 2018 Friends Science Publishers

Keywords: NELFE; PCR–SSCP; PCR–RFLP; Polymorphism; Litter size; Sheep

Introduction

Reproduction efficiency is an important economic trait in livestock production. In particular, litter size, as an important determinant of sheep reproductive capacity, is of great economic significance to the sheep industry (Nadri *et al.*, 2016; Shamim *et al.*, 2016). Litter size in sheep was increased by direct selection, but the gains have not been very large because of its low heritability (0.1–0.15) (Tosh and Kemp, 1995). However, molecular genetics can overcome this traditional selection limitation and offers a novel opportunity to improve reproductive traits, because analysis of genetic variation at the DNA level can potentially detect the individual genes that influence reproductive traits. Identification of litter size-associated polymorphism and DNA markers can lead to genetic improvement through the implementation of marker-assisted selection (MAS) by breeders (Javanmard *et al.*, 2011).

Recently, several genetic markers associated with litter size have been identified in different sheep breeds, including *FecB* (Hudson *et al.*, 1999), *GDF9* (Galloway *et al.*, 2000), *BMP15* (Hanrahan *et al.*, 2004), *IGF1* (He *et al.*, 2012), *ESR* (Ozmen *et al.*, 2012), *KISS1* and *GPR54* (Chu *et al.*, 2012). However, most of these genes affect specific sheep breeds, and are uncommon among all sheep breeds; therefore, further research is needed to identify candidate genes in sheep breeds of interest. Negative elongation factor (NELF) is a multiprotein complex composed of four subunits (A, B, C or D and E), and has been shown to be physically associated with RNP polymerase II and DRB sensitivity-inducing factor to induce transcriptional pausing (Narita *et al.*, 2003). Previous research revealed that NELFE played a pivotal role in *Drosophila* embryogenesis (Wang, 2008; Wang *et al.*, 2010), and was maternally inherited and recruited to gene promoters prior to transcription. NELFA, NELFB, and NELFE knockout resulted in adverse symptoms, such as embryonic cell death, slowness of growth, and apoptosis increase in deficient mouse embryos,

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particularly during implantation (Narita *et al.,* 2007; Amleh *et al.,* 2008; Sun *et al.,* 2011). Interestingly, transcription regulation by NELF is continuously involved in the control of hormone production and may contribute to neuroendocrine cell differentiation (Fujita *et al.,* 2010). In addition, NELFE was suggested to be involved in cell cycle regulation and play important roles in cell cycle progression and cell proliferation (Sun and Li, 2011). Normal cell cycles and proliferation are well known to be necessary for follicular development and normal growth (Wu *et al.,* 2015). The available evidence indicates that NELFE may be involved in cell cycle regulation, embryonic development, and growth; thus, it is a desirable candidate gene for MAS.

In China, two indigenous sheep breeds, Small-tail Han and Hu sheep, are widely reared and are well known for their precocious puberty and high prolificacy (Yan *et al.,* 2005); mean litter sizes for Small-tail Han and Hu sheep were 2.61 and 2.29, respectively, based on field data (Tu, 1989; Yue, 1996). Therefore, genetically improved reproductive performance for these two sheep breeds is crucial. To find DNA markers for MAS of Small-tail Han and Hu sheep, we screened NELFE single nucleotide polymorphism (SNPs) using DNA sequencing, polymerase chain reaction-single strand conformation polymorphism (PCR–SSCP) and polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) technologies.

Materials and Methods

Animal Care

All experimental procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Lanzhou University.

Sample Collection, DNA Extraction, and DNA Pooling Preparation

Venous jugular blood samples, derived from 318 Small-tail Han ewes and 275 Hu ewes (Jinchang Zhongtian Sheep Industry Co. Ltd., Gansu, China) were collected using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood using a standard phenol–chloroform method (Sambrook and Russell, 2006), and then dissolved to 50 ng/μL in ddH_2O and stored at −20°C until analysis. Subsequently, a total of 20 DNA pools were constructed, including 10 for Hu sheep and 10 for Small-tail Han sheep. Each Hu sheep pool contained DNA of 10 individuals (10 μL/individual) from ewes that gave birth to a singleton (n=2), twins (n=2), triplets (n=2), quadruplets (n=2), and quintuplets (n=2) per parity. For Small-tail Han sheep, each pool was a mixture of DNA (10 μL /individual) from 10 ewes that delivered a singleton (n=3), twins (n=3), quadruplets (n=2), and quintuplets (n=2) per parity.

PCR Primer Design

As shown in Table 1, seven primer pairs (P1- P7) were designed based on the DNA sequence of the sheep NELFE (NCBI Accession No. NC_019477.2) using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA). All primers were synthesized by Shanghai Sangon Bio. (Shanghai, China). Primer sequences, amplified regions, annealing temperatures, and product size are summarized in Table 1.

Ovine NELFE SNP Scanning and Genotyping

PCR amplification of seven primers was carried out using the 20 DNA pools as templates. PCR was performed on a 25 μL volume that contained 12.5 μL of 2× Easy Taq PCR Super MIX (TransGen Biotech, Beijing, China), 0.5 μL of 10 μmol/μL of each primer, 10.5 μL double distilled water and 1 μL of 50 ng/μL genomic DNA. The PCR amplification procedure of each primer was as follows: pre-denaturation at 94°C for 5 min; 35 cycles of denaturing at 94°C for 30 s, annealing for 30 s (Table 1), extension at 72°C for 30 s; final extension at 72°C for 10 min; and storage at 4°C. PCR products of each DNA pool for each primer were sequenced using an ABI3700 sequencer. The sequencing results were manually analyzed using Chromas (Technelysium Pty Ltd., Helensville, Australia) and Lasergene (DNASTAR Inc., Madison, WI). Based on the sequencing results, putative polymorphism at P1–P7 loci were genotyped for each individual using PCR–RFLP and PCR–SSCP methods.

Based on DNA pooling sequences, one *XcmI* site for the P3 locus was identified, and PCR–RFLP was carried out by incubating a mixture of 4 μL of P3 PCR products, 4.5 μL of distilled water, 1 μL of enzyme buffer (10×), and 0.5 μL of *XcmI* (New England Biolabs, Beverly, MA) overnight at 37°C. The enzyme digestion of PCR products was separated by electrophoresis on 1.5% agarose gels with DL2000 DNA marker.

Additionally, another polymorphism at the P2 locus was genotyped by PCR–SSCP. PCR–SSCP was performed as follows: 10 μL of P2 PCR products were mixed with 10 μL of denaturing solution (95% formamide, 25 m*M* EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue), denatured at 98°C for 10 min, and then immediately put on ice for 30 min. The denatured DNA was subjected to PAGE in 1× TBE buffer and constant voltage (180 V) for 2.0–2.5 h. The gel was stained with 0.1% silver nitrate. For both PCR–RFLP and PCR–SSCP, one PCR product for each band pattern was sequenced in both directions using an ABI 3700 sequencer.

Statistical Analysis

Population genetic parameters, including Hardy–Weinberg equilibrium, genetic homozygosity (Ho), genetic heterozygosity (He), effective number of alleles (Ne), and

polymorphic information content (PIC) were calculated based on the protocols described previously (Moravčíková *et al.,* 2013). The association of NELFE with litter size was determined using a GLM in SAS 9.2 (SAS Inst. Inc., Cary, NC), and a linear model was implemented as follows:

$$y_{ijlm} = \mu + g_l + b_m + e_{ijlmn}$$

Where, y_{ijlm} is litter size, μ is the population mean, g_l is the genotype effect, b_m is the breed effect, and e_{ijlmn} is the random residual. Correlations and differences with $P < 0.05$ were considered significant.

Results

Identification of Ovine NELFE Genetic Variation Parameters

According to the DNA sequencing for P1-P7 loci using DNA pool as templates, two putative SNPs were identified in P2 and P3 loci, which can be genotyped using PCR-SSCP and PCR-RFLP technologies, respectively. For the P2 locus, combination SSCP analysis and sequencing results showed that a polymorphism, NC_019477.2:g.454T>C, was identified in intron 1 of *NELFE*, and three genotypes (TT, TC, and CC) were found in both Hu and Small-tail Han sheep (Fig. 1). For the P3 locus, combination RFLP analysis and sequencing results showed that a mutation, NC_019477.2:g.883A>C, was identified in intron 2 of *NELFE*, and two genotypes (AA and AC) were found in both Hu and Small-tail Han sheep (Fig. 2). Within the P3 locus, only four individuals had the heterozygous genotype (AC), and the remaining 589 individuals had the homozygous genotype (AA). Because the g.883A>C mutation frequency was too low and minor allele frequency was smaller than 0.01 (MAF=0.003), thus locus was not considered a polymorphic locus and not used for subsequent association analysis. In the ovine NELFE g.454T>C locus, TC was the dominant genotype, and C was the dominant allele (Table 2). In addition, Ho, He, Ne, and PIC for Hu sheep were 0.40, 0.60, 2.50, and 0.41, respectively, and 0.36, 0.64, 2.79, and 0.41 for Small-tail Han sheep, respectively (Table 3). Moreover, the frequencies of the P2 locus for Hu and Small-tail Han sheep were analyzed using a χ^2 test, and it was shown that the P2 locus for Hu sheep did not deviate from Hardy–Weinberg equilibrium (P=0.08), Similar to Small-tail Han sheep, it was not deviate from Hardy–Weinberg equilibrium (P=0.06). Additionally, heterozygosis analysis of ovine NELFE g.454T>C locus showed that two sheep breeds were in a high degree of heterosis (Table 3) and significant frequency differences between different genotypes was present in two sheep breeds ($P < 0.05$).

Association of NELFE Polymorphism with Litter Size

The association analysis of ovine NELFE g.454T>C with litter size was shown in Table 4. The results revealed that ewes carrying TC genotype (1.92) delivered significantly more lambs that ewes with TT (1.67) and CC (1.77) genotypes (P=0.02), indicating that ewes with TC genotype had 0.25 ($P < 0.05$) or 0.15 ($P > 0.05$) more lambs than those with TT or CC genotypes.

In order to investigate the effect of ovine NELFE g.454T>C on litter size in each breed, the association analyses were conducted in Hu and Small-tail Han sheep independently. In general, the results showed a similar tendency to combined analysis that litter size in an order of TC>CC>TT in both Hu sheep and Small-tail Han sheep. In Hu sheep, ewes with genotype TC had 0.39 ($P < 0.05$) or 0.27 ($P < 0.05$) more lambs than those with genotypes TT or CC, respectively. In Small-tail Han sheep, there was no significant difference in litter size among the three genotypes ($P > 0.05$), indicating that the significant association in combined breeds analysis was mainly driven by Hu sheep.

There was a tendency for reproductive performance across the three genotypes to follow the pattern

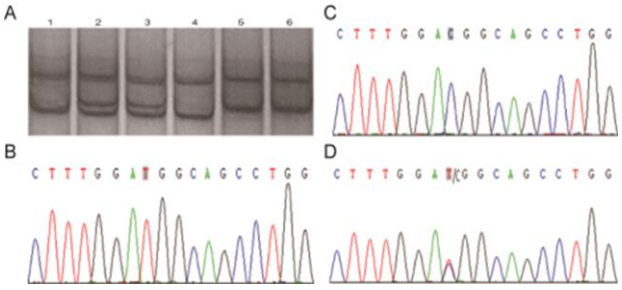

Fig. 1: SSCP analysis and partial sequence comparison of the ovine NELFE P2 locus. (A) Lanes 1 and 4 represent the TT genotype; lanes 2 and 3 represent the TC genotype; and lanes 5 and 6 represent the CC genotype. (B) TT genotype sequencing result. (C) CC genotype sequencing result. (D) TC genotype sequencing result

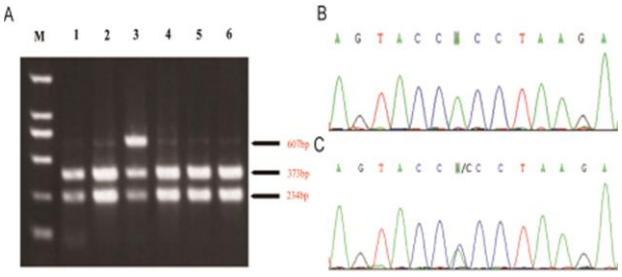

Fig. 2: Band patterns of ovine NELFE digested with XcmI, and partial sequence comparison of the ovine NELFE P3 locus. (A) Lanes 1, 2, 4, 5, and 6 represent the AA genotype; lane 3 represents the AC genotype; and lane M represents the DL2000 DNA marker (100, 250, 500, 750, 1000, 2000 bp). (B) AA genotype sequencing result. (C) AC genotype sequencing result

Table 1: Primer information and PCR conditions used in this study

Loci/Gene	Primer name	Primer sequences (5'–3')	gene region	Tm (°C)	Size (bp)
P1	NELFE1F	F:CGGAGGAAAGGTCACAGGT	Exon 1 and part intron 1	56	483
	NELFE1F	R:GCAGAGGAAGCAGTGGAAC			
P2	NELFE2F	F:TTGCCCCTTTCGTTGCTC	intron 1	58.5	353
	NELFE2R	R:TTTCTCCACTGTCACCGCC			
P3	NELFE3F	F:AAAGGTGAGGGAGTGTGTGTGGG	intron 2	54	607
	NELFE3R	R:GGCAGTGTTTAGTGTTTTCAATG			
P4	NELFE4F	F:CTGTTGTAATGGGCTCTC	Part intron 2 and exon3 and intron3	59.5	1054
	NELFE4R	R;CTGATGGCTCCTGACTTC			
P5	NELFE5F	F:GCCATCAGTGCCATCAAG	Exon4 and exon5 and exon6 and intron 4, intron 5, intron 6	57.5	968
	NELFE5R	R:GGCGAGCAGCAGACAGTG			
P6	NELFE6F	F:TGCCCGAAAACAGCCCAT	Exon10 and intron 10	52	959
	NELFE6R	R:TGAACTCCCTTGTGCCTACTC			
P7	NELFE7F	F:AGGAGTAGGCACAAGGGAGTT	intron 10 and Exon11	53	865
	NELFE7R	R:CACATCAACCTCTACCACG			

P1–P7 represent 11 exons and 10 flanking sequences of ovine NELFE. F indicates forward primers, whereas R indicates reverse primers. Tm indicates Annealing Temperature

Table 2: Genotype and allele frequency distributions of ovine NELFE g.454T>C in two Chinese indigenous sheep breeds

Breed	Number of ewes	Genotype distribution			Allele frequencies		H-W ($\chi 2$)
		TT	TC	CC	T	C	
HS+SHS	593	0.169	0.622	0.209	0.480	0.520	130.00
HS	275	0.156	0.600	0.244	0.456	0.544	61.18
SHS	318	0.179	0.642	0.179	0.500	0.500	69.74

HS represent Hu sheep; SHS represent Small-tail Han sheep

Table 3: Heterozygosis results for ovine *NELFE* g.454T>C of two Chinese indigenous sheep breeds

Breed	H_O	He	Ne	PIC
HS	0.400	0.600	2.500	0.412
SHS	0.358	0.642	2.789	0.412

HS represent Hu sheep; SHS represent Small-tail Han sheep

Table 4: Least squares means and standard deviations for litter sizes associated with different ovine NELFE g.454T>C genotypes of two Chinese indigenous sheep breeds

Breed	Genotype（mean ± SD）			*P*-value
	TT	TC	CC	
HS+SHS	1.67 ± 0.782^{a}	1.92 ± 0.823^{b}	1.77 ± 0.675^{ab}	0.022
HS	1.61 ± 0.929^{a}	2.00 ± 0.917^{b}	1.73 ± 0.617^{a}	0.008
SHS	1.72 ± 0.648	1.85 ± 0.730	1.82 ± 0.735	0.488

HS represent Hu sheep; SHS represent Small-tail Han sheep. Values with the same superscript letter are not significantly different ($P > 0.05$), whereas values with different superscript letters are significantly different ($P < 0.05$)

TC>CC>TT. Analysis of each breed separately showed that the genotype effect on and trend of TC>CC>TT in Hu sheep litter size was consistent with that of the combined breed analysis (HS+SHS). At the *NELFE* locus, Hu ewes with genotype TC had 0.39 ($P < 0.05$) or 0.27 ($P < 0.05$) more lambs than those with genotypes TT or CC, respectively. In Small-tail Han sheep, there was no significant difference in litter size among the three genotypes ($P > 0.05$). However, it is worth mentioning that there is a TC>CC>TT tendency in Small-tail Han sheep.

Discussion

Litter size is an important factor that can improve sheep production, and is influenced by many genetic and environmental factors. Therefore, it is very important to find molecular markers for the selection of ewes with high fertility. A large number of evidences supported that NELFE may play important roles in embryonic development, mammalian fertilization, cell cycle regulation, and growth in model animals, including Drosophila (Wang, 2008; Wang *et al.,* 2010) and mouse (Narita *et al.,* 2007; Amleh *et al.,* 2008; Sun *et al.,* 2011).

Many studies have previously analyzed NELFE polymorphism and function; however, the vast majority have focused on the influence of NELFE on mouse embryonic development (Loones *et al.,* 2000; Narita *et al.,* 2007; Amleh *et al.,* 2008) and human disease (Lévi-Strauss *et al.,* 1988; Speiser and White, 1989; Forbes

and Trowsdale, 1999; Shi *et al.,* 2003). Few studies have evaluated ovine NELFE polymorphism or their effect on ovine litter size. This is the first study to show an association between litter size and a polymorphism in the intron 1 region of ovine NELFE (SNP g.454T>C; Table 4). It was found that heterozygotes (TC) were dominant in both sheep breeds, and TC individuals showed better reproductive performance, which is consistent with several previous findings (Wang *et al.,* 2003a; Fan *et al.,* 2011; Yue *et al.,* 2011). Thus, this locus can be serviced as a molecular marker for increasing sheep litter size after further validation in a larger scale. Table 4 shows that heterozygous Hu ewes tended to produce larger litter sizes than homozygous ewes, which is consistent with genotype frequency distribution.

In this study, association analysis between ovine NELFE g.454T>C and litter size reached to significant level ($P < 0.05$) only in Hu sheep, while not in small-tail Han sheep ($P > 0.05$). It is speculated that this SNP may have larger effect in Hu sheep. Similar to *FecB*, it was found in Booroola Merino, and only showed genetic effect on specialized sheep breeds but not on common breeds. Generally, the current study indicated that ewes with heterozygote produced larger litter size than those with homozygote, which is consistent with classic theory that high heterozygosis is negatively correlated with inbreeding recession and tends to result in high viability and productivity. It was previously reported that there was a significantly positive correlation between genetic heterozygosis and birth weight and litter size in pigs (Fu *et al.,* 2010; Wang *et al.,* 2015) and mice (Mcgloughlin and Cunningham, 1976). In *FecB*, individuals with the B+ genotype had greater litter sizes than individuals with other genotypes (Wang, 2003b) in Merino sheep. Zamani (2015) found that the average litter size of ewes with the AG genotype of BMP15 gene (1.56) was significantly ($P < 0.01$) higher than ewes with the GG genotype (1.08). Similar conclusions were found in many related studies (Chu *et al.,* 2007, 2011; Shi *et al*., 2011). Moreover, other studies have shown that BMP15 is crucial to animal reproduction and found that heterozygous individuals tended to have high reproductive performance, and homozygote fecundity was impaired, because the initial stage of follicular development was blocked (Hanrahan *et al.,* 2004; Bodin *et al.,* 2007; Martinez-Royo *et al.,* 2008; Monteagudo *et al.,* 2009).

It is unknown that the molecular mechanism behind the effect of intronic variation (ovine NELFE g.454T>C) on litter size Hu ewes. Previous studies showed that intronic SNPs can enhance (Chang, 2000) or inhibit gene expression (Beauchamp *et al*., 1998). Moreover, it was previously shown that eukaryotic introns can participate in alternative splicing events, which changes the coded protein (Hirschey *et al*., 2010).

Conclusion

In this study, one SNP (g.454T>C) and one rare mutation (g.883A>C) were identified in intron 1 and intron 2 within NELFE gene according to DNA pooling sequence combined with PCR-SSCP and PCR-RFLP methods. Association analysis of ovine NELFE g.454T>C with litter size revealed that Hu ewes with TC genotype had 0.39 ($P < 0.05$) and 0.27 ($P < 0.05$) more lambs than those with TT and CC genotypes, while this significant association was not observed in Small-tail Han sheep ($P > 0.05$), which could be used in marker-assisted to increase litter size in Hu sheep, and thus improve considerable economic value to sheep producers.

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