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Analysis of *Fcgrt* gene polymorphism in indigenous Chinese sheep and its association with colostrum IgG concentration

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ABSTRACT. The neonatal Fc receptor (FcRn) plays an important role in regulating IgG homeostasis in the body and passive protection to the offspring. Changes in FcRn expression levels caused by genetic polymorphisms of *Fcgrt*, which encodes FcRn, may lead to inter-individual differences in colostrum IgG levels in sheep. In this study, we sequenced the FcRn partial heavy chain from 179 sheep from Xinjiang Province, China, and detected the differences in colostrum IgG levels and *Fcgrt* genotypes to identify the correlation between the *Fcgrt* genotype and colostrum IgG levels in 4 sheep breeds. The DNA sequencing of a 680-bp fragment of the *Fcgrt* gene revealed various patterns depending on the single-strand conformation in the Suffolk breed. Sequencing analysis revealed a total of 3 patterns, AA, BB, AB, in this fragment, among which the absence of AB and BB genotype acted as a marker for breed identification and characterization, while

the AA genotype was shared by Suffolk and 3 other breeds. The only allele found in all 4 breeds was allele A, indicating that natural selection may be favoring the AB and BB genotypes in general and B allele in particular, as the colostrum IgG concentration was relatively higher in the Suffolk breed compared to the other 3 breeds.

Key words: Colostrum; *Fcgrt*; IgG; Genetic polymorphism; Sheep

INTRODUCTION

Maternal immunoglobulin (IgG) is transported across the neonatal intestinal epithelium within the first 24 h of life in lambs, which obtain maternal antibodies solely from colostrum (Besser et al., 1988a). IgG enters the blood circulation via the thoracic duct after traveling through the lymphatics (Besser et al., 1988b); the neonatal Fc receptor (FcRn) plays a predominant role in this process. FcRn, as a novel member of the major histocompatibility complex class I protein family, is evolutionarily distinct from all other Fc receptors (Raghavan and Bjorkman, 1996; Simister and Ahouse, 1996). FcRn was found to bind to IgG and mediate the transmission of IgG from the mother to the fetus perinatally in rodents and humans (Rodewald and Kraehenbuhl, 1984; Israel et al., 1997). It also plays a central role in the protection of serum IgG from catabolism (Junghans and Anderson, 1996). Both the conservation and bioavailability of IgG at all stages of mammalian life are attributable to FcRn (Roopenian and Akilesh, 2007; Ward and Ober, 2009).

The expression levels of FcRn directly correlate with serum IgG concentrations (Brambell et al., 1964). In murine lactating mammary glands, FcRn appears to function as a recycling receptor that transports IgG away from the milk glands (Cianga et al., 1999). In sheep, immunohistological evidence suggests that FcRn is differentially localized in mammary acinar epithelial cells before and after parturition (Mayer et al., 2002). Given the key role of FcRn in IgG and albumin homeostasis and transcytosis, and because the genotype is one of the numerous factors contributing to the failure of passive IgG transfer in sheep (Norman et al., 1981), we hypothesized that there are naturally occurring allelic variants of FcRn with functional consequences. Understanding the transcriptional regulation of the sheep *Fcgrt* gene and how genetic polymorphisms in the *Fcgrt* gene affect receptor expression will help identify alleles that contribute to higher colostrum IgG concentration.

MATERIAL AND METHODS

Animals

A total of 179 female sheep, comprising 4 well-known milch breeds, Chinese Merin (N = 54), Suffolk (N = 51), Romney Hills (N = 42), and BELTEX (N = 32), were maintained at 4 different livestock farms in Xinjiang Province, China. The Chinese Merin samples were collected from the farms of the Xinjiang Production & Construction Group, Shihezi. Suffolk samples were collected from the Key Laboratory of Tarim Animal Husbandry Science and Technology, Xinjiang Production & Construction Group, Tarim. Romney Hills samples were collected from a rural Carla Kurt sheep farm, Carla Kurt, Xinjiang. BELTEX samples were collected from a northern Aksu sheep farm, Aksu, Xinjiang. Samples were collected randomly

from these farms and all samples were used for *Fcgrt* gene polymorphism analysis and association studies of *Fcgrt* gene polymorphisms to determine IgG concentration in colostrum.

Collection of blood samples and colostrum samples

Approximately 2 mL blood was collected from the jugular vein of each animal into a sterile polypropylene vial containing 3% EDTA. The vials were stored on ice and gel cool packs after thorough mixing and were transported to the laboratory and stored at -20°C until DNA isolation. The colostrum samples of 5 mL were collected from the udder of each animal in a sterile polypropylene vial at 48 h after parturition. The vials were kept on ice and gel cool packs and were transported to the laboratory together with the blood samples and stored at -20°C until IgG concentration measurement.

Isolation of genomic DNA and colostrum IgG

For the cloning and sequencing of sheep *Fcgrt* (680 bp), genomic DNA was extracted using a DNA extraction kit (Tiangen Technologies Inc., Beijing, China) from the leukocytes of blood samples. Isolated DNA samples were stored at -20°C until further analysis. IgG in the colostrum was purified by centrifugation at 20,000 *g* for 5 min, after adding albumen precipitation buffer solution into the colostrum and vortexing to mix for 5 min. The supernatant was isolated into a new sterile polypropylene vial and stored at -20°C for high-performance liquid chromatography (HPLC) analysis.

Polymerase chain reaction (PCR) amplification of the *Fcgrt* gene

A specific primer pair was designed based on the bovine FcRn α-chain sequence (Mayer et al., 2002) using the Primerprimer 5.0 software (F: GAGAGATTTGAAGCCCAAGGC; R: AGTACCTGAGCTACAATAACCTG) and standard PCR protocols. A fragment of 680 bp spanning over part of exon II, intron II, and exon III was amplified using the forward and reverse primers. PCR was performed in a 25-μL reaction system using a PCR kit according to the manufacturer (reagents were purchased from Tiangen). The amplification was carried with 3 min denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and 5 min at 72°C. The PCR was performed in a Veriti 96-well thermal cycler (Applied Biosystems, Inc., Foster City, CA, USA).

Nucleotide sequence analysis of the *Fcgrt* gene

Polymorphisms in *Fcgrt* were screened in the fragment being examined using DNA sequencing. After amplification, a 20-μL sample from each PCR product was subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide. The PCR products were examined with respect to a 1500-bp DNA ladder (Tiangen). The agarose gel was then scanned under UV light with Benchtop 3UV Transilluminator (Analytika Jena AG, Jena, Germany), and a single band at 680 bp in each lane was observed and cut with a rubber-cutting knife. The bands were placed into a new sterile polypropylene vial and stored at -20°C for DNA purification. PCR products were then purified using the SanPrep PCR Purification Kit (Sangon Biotech Co., Ltd., Shanghai, China) according to manufacturer protocols. The purified gene

was inserted into a PMD19-T vector. Ligation of the *Fcgrt* cloning insert into the cloning site within PMD19-T was carried out by incubating 100 ng (3.5 μL) annealed dsDNA with 0.5 μL linearized vector with 5 U T4 DNA ligase at 4°C overnight in 5 μL 2X Rapid ligation buffer. The product was transformed into chemically competent DH-5α cells (Takara, Ltd.). The cells were then plated on LB dry plates supplemented with 10% ampicillin and cultured at 37°C overnight, then the positive clones were selected and the plasmids were extracted using a plasmid extraction kit (Takara, Ltd.). Hybridized plasmids were tested by agarose gel by single-endonuclease digestion with *Bam*HI, and double-endonuclease digestion with *Bam*HI and *Xho*I. The plasmids were then sent to Sangon Biotech Co., Ltd., for standard double-strand DNA sequencing.

Colostrum IgG concentration detection by HPLC

IgG concentrations in the collected colostrum samples were estimated using HPLC with a 1-mL HiTrap Protein G HP column (No. 17-0404-01; GE Healthcare Bio-Sciences AB, Little Chalfont, UK). Bovine serum IgG used for calibration was reagent grade (95% SDS-PAGE) (No. I-5506, Sigma Aldrich, St. Louis, MO, USA). Bovine serum IgG for fortification was laboratory grade (No. IGGL, ICP Biotech, Auckland, New Zealand). Colostrum (1 mL) was combined with an equal volume of sedimentation buffer and centrifuged at 20,000 *g* for 5 min. The supernatant was collected and filtered through a 0.45-μm microfiltration membrane. The filtrate was collected for HPLC analysis. The gradient dilution was as follows: 0-0.5 min, 100% loading buffer, flow rate 1.0 mL/min, increase to 2 mL/min after 1 min; 1.0-1.5 min, 100% washing buffer, until 4 min; 4-5 min, loading buffer for 2 min, equilibrium was reached before the next insertion, and the insertion volume was 100 μL.

Statistical analysis

Genotype differences among the 4 sheep breeds and individuals in each breed were analyzed using multiple sequence alignment with DNAMAN (Version 6.0). The difference in IgG concentrations in colostrum among the 4 sheep breeds was analyzed by one-way analysis of variance with the SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

DNA polymorphism analysis of the *Fcgrt* gene

The DNA sequence of the 680-bp fragment of the *Fcgrt* gene revealed various patterns depending on the single-strand conformation in the Suffolk breed (Figure 1). Each animal in this breed exhibited a specific genotype. Sequencing analysis revealed a total of 3 patterns, AA, BB, AB, in this fragment with 2 alleles, A and B. Among these genotypes, the absence of the AB and BB genotype was used as a marker for breed identification and characterization, while the AA genotype was shared by Suffolk and the other 3 breeds, including Chinese Merin, Romney Hills, and BELTEX (Figure 2). The only allele found in all 4 breeds was the A allele, indicating that natural selection may be favoring the AB and BB genotypes in general and B allele in particular, as the colostrum IgG concentration was relatively higher in the Suffolk breed compared to the other 3 breeds. Because there is a lack

of related genotype polymorphism information in the literature, these findings could not be validated using previous studies.

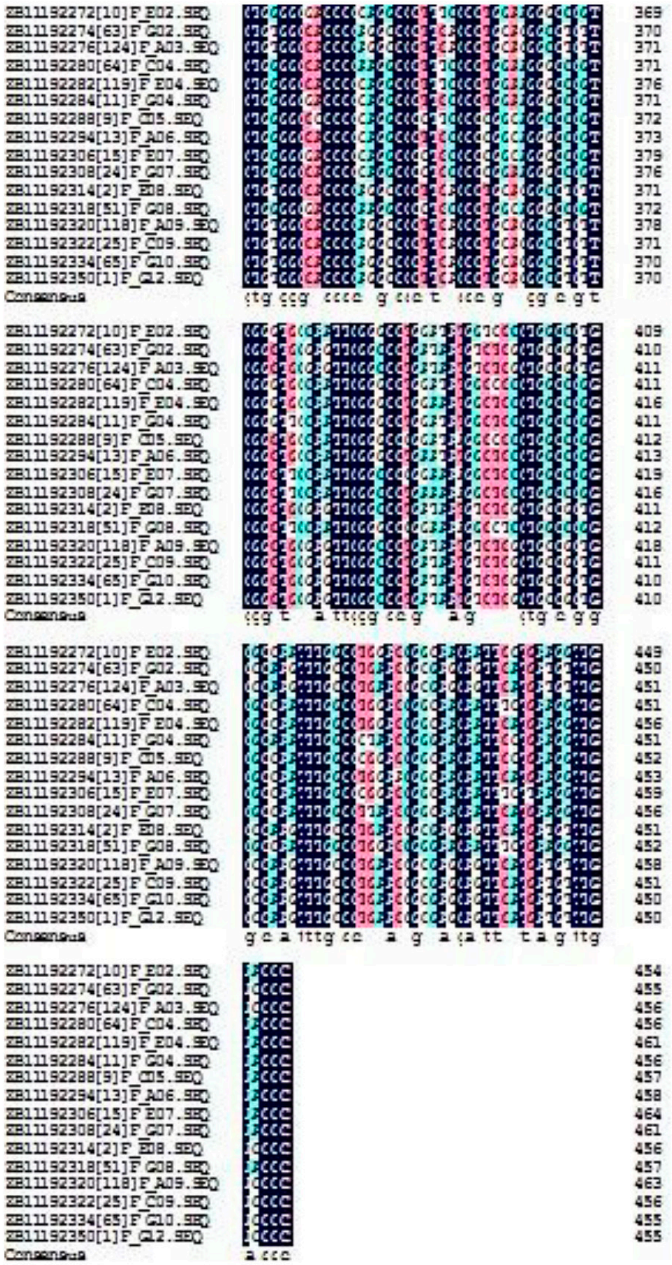

Figure 1. Sequence BLAST results of Suffolk sheep (partial).

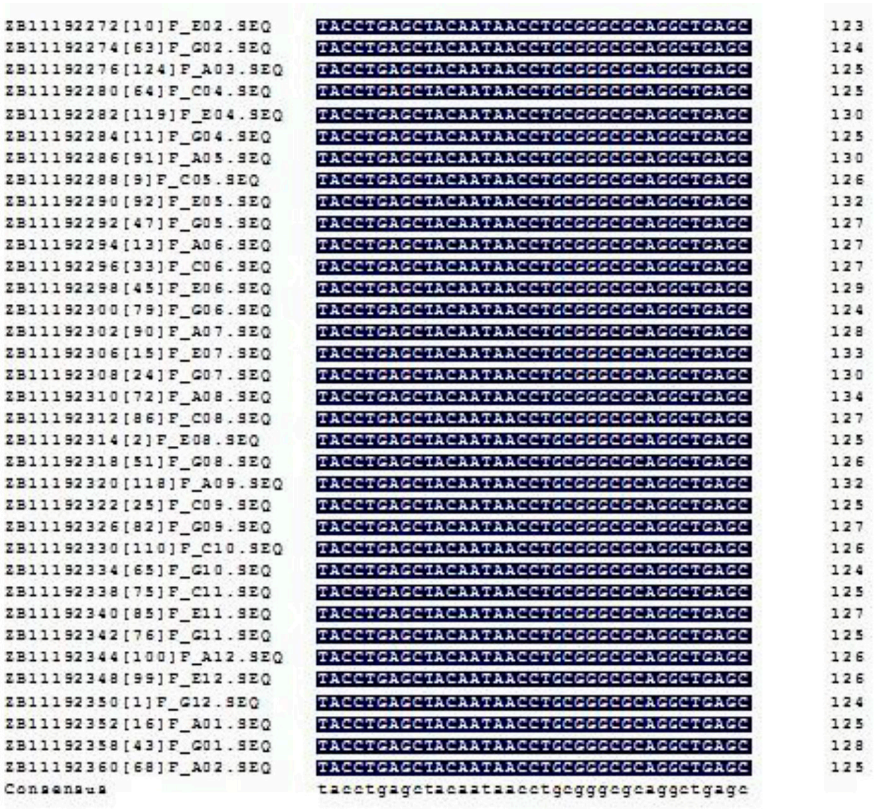

Figure 2. Sequence BLAST result of 4 sheep breeds (partial).

For the other species, there was a sufficient number of polymorphisms in the *Fcgrt* gene in the various breeds of sow and dairy cows for examination. Laegreid et al. (2002) investigated the allelic variation of *Fcgrt* and its association with variation of IgG concentration in neonatal calves, and 5 single-nucleotide polymorphisms were identified by sequencing 1305 bp of the *Fcgrt* genomic DNA from a multi-breed panel of 96 cattle and 27 founders of a reference population. Clawson (2004) evaluated haplotypes associated with high or low serum IgG levels in newborn calves. Eight haplotypes were identified by sequencing regions of B2M exons II and IV in a multi-breed panel of 96 beef cattle. Calves homozygous for 1 of the 8 haplotypes (B2M 2.2) were at increased risk of failure of passive IgG transfer. However, Freiberger et al. (2010) found no association between the FcRn promoter polymorphism, both maternal FcRn and of fetal FcRn, and the rate of maternal-fetal IgG transfer by analyzing 103 single fetal samples and 103 paired maternal and fetal samples collected from the umbilical cord blood of full-term neonates.

Colostrum IgG concentration in Suffolk, Chinese Merin, Romney Hills, and BELTEX sheep

For many mammals, the young offspring obtain passive immunity from the colos-

trum; IgG plays a dominant role in this immunoprotective action, with a concentration as high as 101 mg/mL in sheep colostrum. We found that the BB genotype had the highest average (105 mg/mL) IgG concentration, while the AA genotype had the lowest average (85 mg/mL) IgG concentration. IgG concentration is an index of the FcRn expression level and reflects the status of IgG transport (mainly FcRn-mediated) system in the body. These results may have been observed because of the different sample collection periods used. Differences in the colostrum IgG concentration were statistically significant among the various genotypes.

Total IgG colostrum levels and the ratios of IgG content >10%/total samples tested are shown in Figures 3 and 4. The intervarietal differences in total colostrum IgG concentrations were not significant among the 4 breeds ($P > 0.5$; Figure 3), while the differences in the ratios of IgG content >10%/total samples tested are significant ($P < 0.0001$; Figure 4). The colostrum IgG concentration variety within 1 breed ranged from 19.36-3.05% in Chinese Merin, 21.94-2.64% in Suffolk, 20.64-0.73% in Romney Hills, and 21.63-3.27% in BELTEX. This interclass significance can be partially explained by individual differences; however, other factors should be further explored.

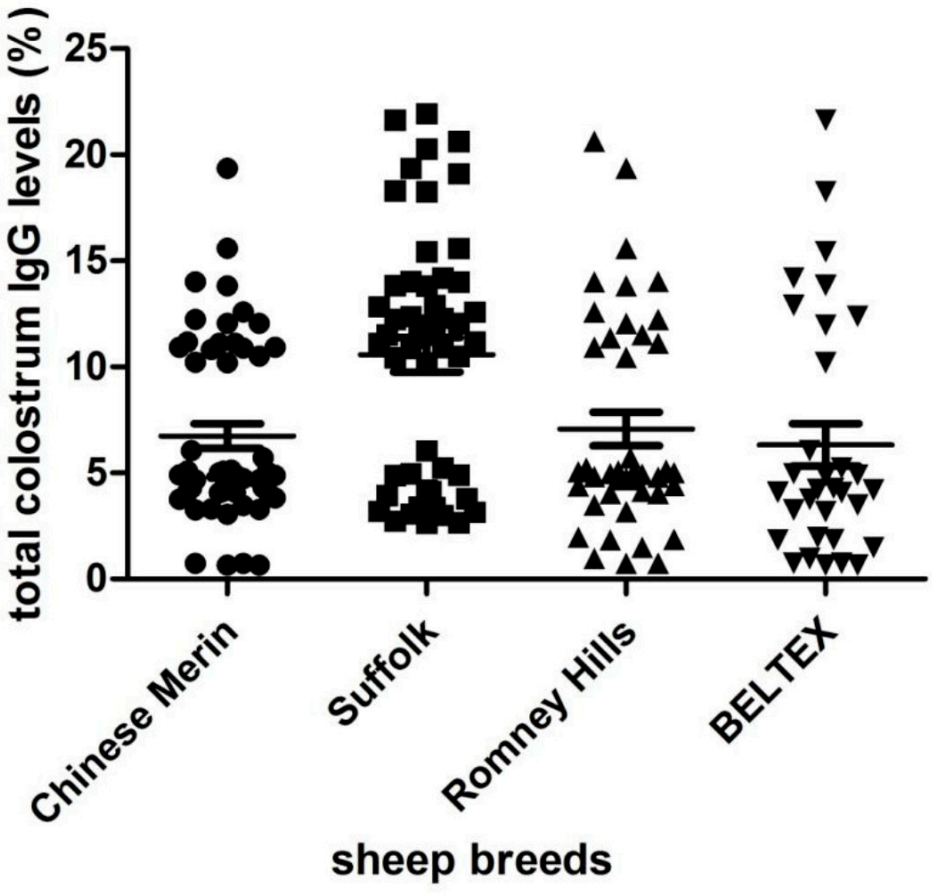

Figure 3. IgG contents in the colostrum of Suffolk, Romney Hills, BELTEX, and Chinese Merin.

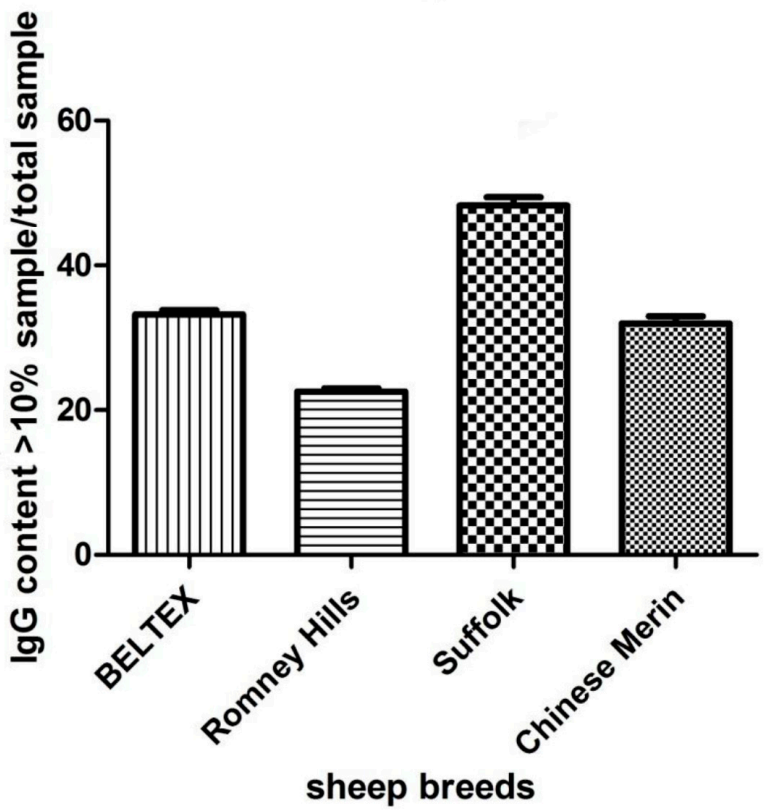

Figure 4. Differences among breeds in samples of IgG relative content >10%.

Nucleotide sequence analysis

Various alleles of the 680-bp fragment of the *Fcgrt* gene were sequenced using Sanger's dideoxy chain termination sequencing method. The sequences obtained were compared with other available sequences from sheep (Accession No. Q8HZV2), and the amplified fragment was confirmed to include exon II, intron II, and exon III. All sequences were aligned using Multiple Sequence BLAST program of the DNAMAN software using Accession No. Q8HZV2 as the corresponding sequence (Figure 1 shows partial data). Based on the alignment of the allelic variants, there are differences in 23 positions among the alleles, of which maximum variations (at 13 locations) were found in the exon II region in contrast to 4 variations in the exon III region. As there is no genotype information available regarding sheep *Fcgrt*'s intronic region, various substitutions in the *Fcgrt* intron II region observed in this study werenot analyzed.

CONCLUSIONS

In mammals, FcRn has prominent maternal and neonatal roles in IgG transfer (Ghetie and Ward, 2000). Among the 4 breeds examined, numerous mutations existed in the *Fcgrt* se-

quence in Suffolk (Figure 1) compared to individuals within the breed and across the breeds, while the other 3 breeds showed few mutations in the genes tested (Figure 2). A possible explanation for this observation is that the Suffolk breed is mainly raised for wool and meat purposes in this area, and its members were crossed with the progeny of other mountain ewes, whereas the other 3 breeds were pure. It is common to have more mutations in a crossbreed compared to pure breeds, even within the same gene.

The presence or absence of a particular genotype observed in this study can be utilized as a marker for breed identification. No significant effect of genotypes was observed on colostrum IgG concentration, although there were some significant differences among the mean values. This may be because the 680-bp fragment of the *Fcgrt* gene may contribute in part to the total variation present. The change in the amino acid sequence of FcRn may influence its binding affinity with IgG, thus influencing the transportation effect of maternal IgG into colostrum.

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