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Research Article

# Identification of SNP Within the Sheep *RXRG* Gene and Its Relationship with Twinning Trait in Sheep

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## Abstract

*RXRG (Retinoic X receptor-gamma)* gene was originally associated with fetal development and reproduction in human beings and animals, which was used to detect genetic variation that was associated with growth, reproduction, metabolism trait selection and breeding. The aim of this study was to detect *RXRG* gene mutation of the exon 2 and its association with twinning trait in 313 sheep and calculate litter size, genotype frequency in Chinese Merino, Hu and Kazak sheep. Polymorphism of exon 1, exon 2, exon 10 of *RXRG* gene in the study were analyzed by *PCR-SSCP,* which showed that three genotypes of P2 fragment were significantly associated with twinning traits in the analyzed population (P=0.031). Analysis of four groups of sheep showed that there was a predominate gene (B-allele) that have higher twinning rates in these individuals, which indicated that the genotypes can be chosen for twinning according to predominate genotype. In conclusion, our results strongly suggest that polymorphisms of the *RXRG* gene could be a new choice for sheep breeding and genetics through marker-assisted selection (MAS).

***Keywords:*** *Sheep, Retinoic X receptor-gamma gene, Twinning trait, PCR-SSCP*

# Koyun *RXRG* Geninde SNP İdentifikasyonu ve İkizlik İle İlişkisi

## Özet

*RXRG (Retinoik X reseptör-gama)* geni ilk olarak hayvan ve insanlarda fetal gelişim ve üreme ile ilişkilendirildi ve büyüme, üreme, metabolizma özellik seleksiyonu ve üreme ile ilgili genetik varyasyonu belirlemek için kullanıldı. Bu çalışmanın amacı, ekzon 2 *RXRG* gen mutasyonunu ve 313 koyunda ikizlik ile ilişkisini belirleyerek Çin Merinos, Hu ve Kazak koyunlarında yavru sayısı ve genotip frekansını hesaplamaktır. Bu çalışmada *RXRG* geni exon 1, exon 2 ve exon 10 polimorfizmleri *PCR-SSCP* ile analiz edildi ve incelenen popülasyon içerisinde P2 parçacığının üç genotipinin anlamlı derecede ikizlik ile ilişkili olduğu belirlendi (P=0.031). Dört grup koyunun analizi sonucunda bir baskın genin (B-allel) bu bireylerde yüksek ikizlik oranına sahip olduğunu ve bu genotipin ikizlik için seçilebileceğini gösterdi. Sonuç olarak, elde ettiğimiz sonuçlar *RXRG* gen polimorfizminin Marker ilişkili seleksiyon (MAS) yoluyla koyun üretiminde ve genetiğinde kullanılabileceğini önermektedir.

***Anahtar sözcükler:*** *Koyun, Retinoik X reseptör-gama geni, İkizlik, PCR-SSCP*

## INTRODUCTION

Most breeds of sheep usually produce one-offspring per year, with a longer lambing interval and lower reproductive efficiency, which greatly restricting the development and production of sheep industry. Meanwhile, it is known that reproductive traits of domestic animals were controlled by a series of relevant reproductive genes that has a low heritability [1], therefore using conventional breeding methods to improve the phenotype of the reproductive efficiency in sheep that is not only time consuming, but also difficult to obtain an ideal production efficiency in the sheep industry.

Retinoic acid, as a fat-soluble small molecule, is the main

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regulator of cell differentiation and tissue morphogenesis, which plays an important role in the process of epithelial cell growth, the maintenance of visual organization, and fetal development and reproduction [2-4]. The pleiotropism of retinoic acid are mediated by retinoic acid receptor *(RAR)* and luteinized X receptor *(RXR)* that are members of the steroid/thyroid hormone receptor superfamily of nuclear receptor proteins [3,5-8]. *RAR* are ligand-controlled transcription factors that function as heterodimers with *RXR* to exert their effects by binding to specific DNA response elements, thus regulating gene expression in target cells [3,6,9-12], which were encoded by 3 different genes, including α, β and γ, and that results in the forming more types of receptors, such as *RARA*, *RARB*, *RARG* and *RXRA*, *RXRB*, *RXRG* [13]. Previous studies showed that *RXRG* gene can be expressed during pregnancy, which has a significant additive effect on litter size in the pregnant females [14]. Similarly, Huang *et al* studied found there are the relationship between genetic variation of *RXRG* and twinning trait in cow that the individuals with AB genotype of *RXRG* gene could produce more twins than those with AA genotype [15].

However, there are few reports on the relationship between *RXRG* gene and twinning trait in sheep. To explore the correlation between the genetic variation of sheep RXRG gene and its relationship with reproductive traits, in this study, genetic variation in 3 exons of *RXRG* gene (Based on the reported *RXRG* in ovis aries gene sequences) and the flanking regions were investigated in 313 sheep by using PCR-SSCP. The relationship between *RXRG* mutations and litter size in sheep was evaluated to examine *RXRG* gene as a candidate gene for sheep litter size traits. Thus, the results of the study would be beneficial to provide a theoretical basis for application on the molecular assisted breeding based on the genetic markers related to the prolificacy of sheep.

# MATERIAL and METHODS

### *Ethics Statement*

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University. All samples were collected in strict accordance with the committee's guidelines.

### *DNA Samples and Lambing Records*

Three hundred and thirteen genomic DNA samples were obtained from healthy ewes by intravenous blood collection in Xinjiang, China. All the sheep in the study aged four years and weighed 46±2.48 kg that were housed individually under the same feeding conditions including *ad libitum* access to *alfalfa* and water. All the sheep were randomly divided into four different groups were named group 1, 2, 3 and 4, respectively, among which group 1 and 2 consisted of 178 China Merino sheep with singletons and 58 China Merino sheep with twins respectively, and group 3 contained 40 Kazak sheep and group 4 includes 37 Hu sheep. Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocol [16]. Besides that, all lambing records of them were obtained from the production records in the sheep farms.

### *Primer Design and PCR Amplification*

According to the reported sheep *RXRG* gene sequences (NC_019458), three pairs of primers (P1, P2, and P3) were designed to amplify the sheep *RXRG* gene. P1 (F: 5′-CCAAAGCCTGTGGGAAACT -3′ and R: 5′- GCGGCATTATGC GTGATT -3′), P2 (F: 5′- GGGGCAACCAGATTGATTCCT -3′ and R: 5′- TCGGCAGCCTTGTCCAC -3′), P3 (F: 5′- AGCCCTGCG TTCTAT -3′ and R: 5′- AGGCGGAGGAGCAT -3′) were separately used to amplify 307 bp, 197 bp and 2204 bp PCR products for exon 1, 2 and 10 respectively. The PCR was performed in a 25 μL reaction mixture containing 0.4 μM of each primer, 200 μM dNTPs, 1×polymerase buffer (including 1.5 mM $MgCl_2$), 1 units of Taq DNA polymerase (Sangon, China) and approximately 100 ng genomic DNA as template. The cycling protocol was 5 min at 95°C followed by 35 cycles of 94°C for 30 s, X°C annealing for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min (X°C was 59, 59, 55°C for P1, P2 and P3 primers, respectively).

### *Single Stranded Conformation Polymorphism (SSCP)*

All PCR products were subjected to SSCP analysis. Aliquots of 2 μL PCR products were mixed with 8 μL loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), denatured by heating at 98°C for 10 min and immediately placed on wet ice. Denatured samples of P1, P2 and P3 were loaded on 10% PAGE gel in 0.5×TBE buffer and constant voltage 140 V for 14-16 h after a pre-run at 220 V for 50 min. The gel was stained by a silver staining method [17].

### *DNA Sequencing Analysis*

The 14 PCR products showed different electrophoresis patterns, which were subcloned to pMD19-T vector (Tiangen, China) and sequenced using a commercial service (Huada, Beijing, China). Nucleotide sequence alignments, translations and comparisons were carried out by using DNAMAN software, respectively.

### *Statistical Analysis*

Genotypic and allelic frequencies and Hardy-Weinberg equilibriums were estimated. The following model was used to analyze the association between different genotypes with twinning trait: $Y_{ik} = \mu + Age_i + Marker_k + e_{ik}$, which $Y_{ik}$ was twinning trait that was measured on each of ikth sheep, μ was the overall population mean, $Age_i$ was type of the ith age, $Marker_k$ was the fixed effect associated with kth genotype and $e_{ik}$ was the random error. And Chi square test for independence in polymorphic loci of the

genotypes distribution in single-twin groups [18]. Statistical analysis was carried out by SPSS for Windows 13.0.

# RESULTS

### *SSCP and Sequence Analysis*

Polymorphisms were found in the P2 fragments by SSCP analysis *(Fig. 1)*. In the P2 fragment, three unique SSCP genotypes were obtained and designated as AA, AB and BB. PCR products also indicated that there were three genotypes were sequenced in both directions in ABI PRISM 377 DNA sequencer. And subsequent sequence comparison revealed there were two SNPs of the P2 fragments in sheep in China, including g.131A > G and g.32G > A, which the former was changed to AA- and AB-genotypes and the latter was changed to BB- and AB-genotypes, however, the polymorphisms weren't found in the P1and P3 fragments by SSCP analysis.

### *Genetic Polymorphism of RXRG Gene in Sheep*

Above all, in our study, it is worth to note that Kazak sheep is a typical single breed, Hu sheep is a typical multiparous breed, however, China merino sheep is a single and twins breed. The genotype frequency and allele frequency of *RXRG* gene in sheep were shown in *Table 1*, BB-genotype frequency of Kazak sheep in the Group 3 was higher than AB and AA, however, as well as the former one of Hu sheep in the Group 4 was also the highest but there were only two genotypes, including BB and AB. Similarly, BB-genotype frequency was higher than AA and AB of China Merino with twinning trait in the Group 2, but AB-genotype frequency was higher than AA and BB of China Merino with singletons trait in the Group 1, all of which showed B-genotype was the predominant allele in all the populations.

### *Genetic Characteristics of loci in Sheep Populations*

Polymorphism information content, population heterozygosity, effective allele number, and Chi-square test of loci in each group were shown in *Table 2*. Polymorphism information content (PIC) of the twins and singleton populations in China Merino sheep was separately 0.328 and 0.345 that was ranged within 0.25-0.50, which indicated that the loci was moderately polymorphic in these two populations. The study results showed that the χ2 value of the loci in China Merino sheep with twins was 6.532 ($P<0.05$), and the χ2 value of the loci in China Merino sheep with singleton was 2.264 ($P>0.05$), which indicated that the mutation loci in China Merino

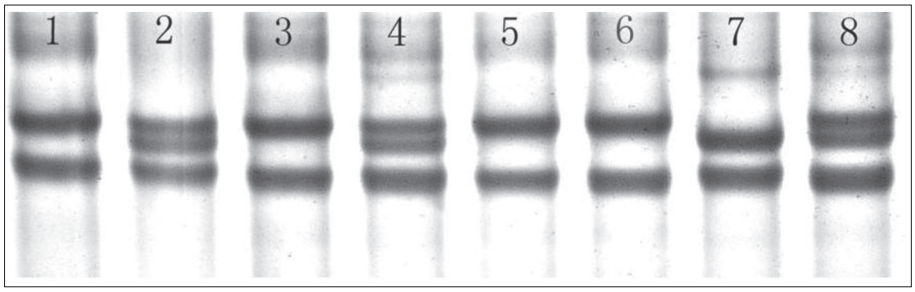


**Fig 1.** SSCP analysis of PCR amplification using P2
7: AA genotype 2, 4, 8: AB genotype 1, 3, 5, 6: BB genotype

***Table 1.*** *The genotype frequency and gene frequency of RXRG gene in China Merino*

| Group No. | Group | No. | RXRG2 Primer | | | | |
|---|---|---|---|---|---|---|---|
| | | | Genotype Frequency | | | Allele Frequency | |
| | | | AA | AB | BB | A | B |
| 1 | China Merino sheep with singleton trait | 178 | 0.084 | 0.494 | 0.422 | 0.331 | 0.669 |
| 1 | China Merino sheep with twinning trait | 58 | 0.155 | 0.276 | 0.569 | 0.293 | 0.707 |
| 2 | Kazak sheep | 40 | 0.150 | 0.400 | 0.450 | 0.350 | 0.650 |
| 3 | Hu sheep | 37 | 0.000 | 0.270 | 0.730 | 0.135 | 0.865 |

***Table 2.*** *Data of Heterozygosities(H), Effective number of Alleles (Ne), Polymorphism information contents (PIC) and $\chi^2$-test*

| Group No. | Group | PIC | H | Ne | $\chi^2$-test |
|---|---|---|---|---|---|
| 1 | China Merino sheep with singleton trait | 0.345 | 0.505 | 1.796 | 2.264 ($P>0.05$) |
| 1 | China Merino sheep with twinning trait | 0.328 | 0.724 | 1.708 | 6.532 ($P<0.05$) |
| 2 | Kazak sheep | 0.336 | 0.643 | 1.747 | 3.436 ($P<0.05$) |
| 3 | Hu sheep | 0.206 | 0.730 | 1.305 | 0.804 ($P<0.05$) |

**Table 3.** *Gene frequency of RXRG in different group by χ2 -test*

| Group | Group | χ2 |
|---|---|---|
| China Merino sheep with singleton trait | China Merino sheep with twinning trait | 8.986 (*P<0.05*) |
| China Merino sheep with singleton trait | Kazak sheep | 2.138 *(P>0.05)* |
| China Merino sheep with singleton trait | Hu sheep | 12.634 (*P<0.05*) |
| China Merino sheep with twinning trait | Kazak sheep | 1.765 (*P>0.05*) |
| China Merino sheep with twinning trait | Hu sheep | 6.668 (*P<0.05*) |

**Table 4.** *Correlation analysis between different genotypes of RXRG*

| Gene Locus | Breed Character | Genotype | Mean ± Std. |
|---|---|---|---|
| RXRG2 | Litter size | AA | 1.375±0.087[a] |
| | | AB | 1.154±0.042[b] |
| | | BB | 1.306±0.041[a] |

sheep with singleton as well as the one in Kazak and Hu sheep were in the state of Hardy-Weinberg Equilibrium, but the mutation loci in China Merino sheep with twins were not in the state of Hardy-Weinberg Equilibrium, which the reason for this latter phenomenon may be that the China Merino sheep with twins were man-made intervention.

### *Genotype Distribution of P2 loci in RXRG in the Different Sheep Population*

The genotype distribution of the polymorphic loci of the RXRG gene was analyzed in four groups using Chi-square independence test. There were significant differences between China Merino sheep with single trait and China Merino sheep with twinning trait and Hu sheep respectively (*P*<0.05), similarly, there were also significant differences between China Merino sheep with twinning trait and Hu sheep (*P*<0.05), however, there weren't significant difference between China Merino sheep with single or twinning trait and Kazak sheep *(Table 3)*.

### *Association Analysis Between the Polymorphism of RXRG Gene and Lambing Traits in Sheep*

Through general linear correlation analysis between different *RXRG* genotypes and the litter size in China Merino sheep, compared with AB-genotype, China Merino sheep with AA-, BB-genotype were significantly higher litter size (*P*<0.05), which showed that AA- and BB-genotypes were more likely to have twins than AB-genotype. However, due to a smaller number of individuals with AA-genotype, which the difference had no statistically significant. Besides that, we also conducted a Chi-square test for independence, which there were significant differences between the individuals with singletons and twins in China Merino sheep (*χ2*=8.986, *P*=0.011) *(Table 4)*.

## DISCUSSION

Genetic characteristics of single nucleotide mutation in this study were analyzed in the exon 2 of *RXRG* gene in sheep. There were three mutation sites of P2 site in *RXRG* that were G, T and A by comparing with the reported *Ovis aries RXRG* sequences, respectively, among which AA-genotype with an A → G mutation in 131bp and BB-genotype with a G → A mutation in 32bp were not cause amino acid changes. However, these mutations were not correlated with protein expression and therefore these mutations had not influenced gene translation [19]. Association analysis between genotype effect and twinning trait in China Merino sheep found that P2 mutation rate was higher, which showed that there were significant differences in the genotype distribution of among singleton and twins groups (*P*<0.05) and the different genotypes have a greater impact on the phenomenon of sheep twins that suggested that *RXRG* could affect the twinning trait in China Merino sheep. In this study, we selected *RXRG* gene in different sheep breeds to study the genotype frequency and gene frequency of *RXRG* gene, such as China Merino sheep, Kazak sheep, Hu sheep, which indicated that B-allele could be the predominant gene in any one of four sheep populations and it might provide a choice for selecting the sheep breeds with twinning trait.

In the previous studies, there were only few reports about the impact of *RXRG* Gene on animal reproductive traits. Messer *et al* found retinoic acid receptor, gamma *(RARG)* gene can be expressed in the critical period of pregnancy in pig, and there would be a higher of litter size in Large White pigs if *RARG* gene was expressed that the average litter size increased by 0.21 pigs [14]. Huang *et al*.[15] found that AB-genotype of *RXRG* gene in the cow would be more likely to produce twins than AA-genotype, and there were significantly different between different genotypes with singleton and twins *(P*=0.0006). Similarly, Guo *et al*. [20] reported that the average litter size in Small Tailed Han sheep with CC-genotype of *RARG* gene were higher than CD-genetype by 0.55 *(P*<0.05), all of which indicated that *RXRG* gene have a significant impact on the fecundity of pigs and cow. Additionally, here it is noteworthy that we also found in our experiment that the mutation site of *RXRG* gene had impact on twinning trait in sheep. There were a higher of litter size in China Merino sheep with P2 sites of AA- and BB-genotypes than AB-genotype that the average litter size increased by 0.22 and 0.15 sheep, respectively *(P*<0.05), and importantly, there was a statistically significant significance that the individuals with BB-genotype was more likely to produce twins than

AB-genotype in sheep. In conclusion, our results strongly suggest that there were a certain correlation between polymorphisms of the *RXRG* gene and twinning trait that could be a new choice for sheep breeding and genetics through *MAS*.

## Competing Interests

There are no potential conflicts of interest.

## Acknowledgements

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