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Identification of SNP Within the Sheep *RXRG* Gene and the Association Analysis with Twinning Trait

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Abstract: *RXRG* (Retinoic X Receptor-γ) gene was originally associated with fetal development and reproduction in human beings and animals. With the rapid development of genetics, more and more attention are being turned to *RXRG* gene mutation and its influence on litter size. *RXRG* gene as a new candidate gene was used to detect genetic variation and that change was associated with growth, reproduction, metabolism traits selection and breeding. The aim of this study was to detect *RXRG* gene mutation of the exon 1-3 and the mutation's association with twinning traits in 313 sheeps and to calculate litter size, genotype frequency in Chinese Merino, Hu and Kazak sheep. In this study, the polymorphism of exon 1, exon 2, exon 10 of *RXRG* gene were analyzed by PCR-Single Strand Conformation Polymorphism (PCR-SSCP) and association analysis 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 predominant gene (*Allele B*) and those populations which carried that allele would have higher twinning rates and that may become a useful molecular instrument for predominant genotype-choosing of twinning trait. These results strongly suggest that polymorphisms of the *RXRG* gene could be a new choice for sheep breeding and genetics through Marker-Assisted Selection (MAS).

Key words: Sheep, retinoic X receptor-γ gene, twinning trait, PCR-SSCP, breed

INTRODUCTION

Most of the fetal sheep usually produce one-offspring per year and this long production cycle with low fecundity greatly limits the sheep industry. Meanwhile, it is known that reproductive traits of domestic animals were controlled by a series of relevant reproductive genes and at the same time sheep has a low heritability (Van Vleck and Gregory, 1996) therefore using conventional breeding methods to improve the phenotype of sheep fecundity is not only time consuming but also difficult to obtain great progress.

Retinoic acid, a fat-soluble small molecule is the main regulator of cell differentiation and tissue morphogenesis and plays an important role in the process of cell differentiation, epithelial cell growth, the maintenance of visual organization and fetal development and reproduction (Wirtanen and Seguin, 2000; Burrage *et al*., 2007; Liu *et al*., 2005). This pleiotropism of retinoic acid are mediated by retinoic acid receptor (Retinoic Acid Receptor, RAR) and luteinized X receptor (Retinoid X Receptor, RXR) which has two types of receptors of transcription factor superfamily (Burrage *et al*., 2007; Chambon *et al*.,1991; Michaille *et al*., 1994; Lohnes *et al*., 1993; Huang *et al*., 2008).

RXR is a protein which can assist retinoic acid receptor to activate and then RXR could become heterodimer with the combination of RAR (RAR/RXR) which will increase the affinity of RAR and response element of retinoid and strengthen RAR transcriptional activity and sensitivity of ligand (Burrage *et al*., 2007; Leid *et al*., 1992, 1993; Michaille *et al*., 1994; Zhang, 2009). RAR and RXR can be encoded from 3 different genes *RXRA*, *RXRB* and *RXRG* and that results in the forming of more types of receptors such as RXRA, RXRB, RXRG and so on (Liu *et al*., 1997b). As a ligand activated transcription factors, *RXRG* gene can be binded to specific response sequences of target genes (*DNA*) and that will play an important role in regulating gene transcription and expression and become the main regulator of cell differentiation and tissue morphogenesis (Lohnes *et al*., 1993).

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Previous studies showed that *RXRG* gene can be expressed during pregnancy which has significant additive effect on litter size of pig and also has a significant impact for some cow breeds. But there are few reports on the relationship between *RXRG* gene and sheep twinning traits. Huang *et al.* (2008) studied the relationship between genetic variation of cow RXRG and twinning trait and found that genotype AB of cattle *RXRG* gene produces more twins than AA genotype. Mu *et al.* (2006) regarded sheep PRLR as a candidate gene of high reproductive rate, studied polymorphism of its intron 2 and found that there are correlation with litter size in small tail Han sheep (Mu *et al.*, 2006). But the relevent research of relationship between *RXRG* gene and sheep twinning trait in the country has not been reported.

In this study, genetic variation in 3 exons of *RXRG* gene (Based on the reported *RXRG* bovine gene sequences) and flanking regions was investigated in 313 sheep using PCR-SSCP. Additionally, the relationship between the sheep RXRG mutations and litter size was evaluated in order to examine *RXRG* gene as a candidate gene for sheep litter size traits.

MATERIALS AND METHODS

DNA samples and lambing records: Three hundred and thirty nine genomic DNA samples were obtained from healthy ewes in Xinjiang, China. All the sheep were randomly divided into three different groups (named group 1, 2 and 3). At 165 Mission of Tacheng there were two groups of sheep. Group 1 contained 236 China Merino, group 2 consisted of 50 Kazak sheep. At Xinhu sheep farm of Xinhu, group 3 include 53 Hu sheep. All the sheep were 4 years old and they were fed freely, Each of them almost reached 46 kg. Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocol (Liu *et al.*, 1997a). Lambing records were measured from the sheep farm production records in 2010.

Primer design and PCR amplification: Based on cattle *RXRG* gene sequences (NC_007301), three pairs of primers (P1, P2 and P3) were designed to amplify the sheep *RXRG* gene. P1 (F: 5'-CCAAAGCCTGTGGGAAACT-3' and R: 5'-GCGGCATTATGCGTGATT-3') was used to amplify 307 bp PCR product for exon 1; P2 (F: 5'-GGGGCAACCAGATTGATTCCT-3' and R: 5'-TCGGCAGCCTTGTCCAC-3') was used to amplify 197 bp PCR product for exon 2; P3 (F: 5'-AGCCCTGCGTTCTAT-3' and R: 5'-AGGCGGAGGAGCAT-3') was used to amplify 204 bp PCR product for partial exon 10 (Table 1). 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 MgCl2), 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 sec, X°C annealing for 30 sec, 72°C for 30 sec with a final extension at 72°C for 10 min (X°C was 59, 59, 55°C for P1, P2 and P3 primers, respectively).

Table 1: Primers of *RXRG* gene

Fragments names	Primer pairs	Notes	Sizes (bp)	Temp. (°C)
P1	F: CCAAAGCCTGTGGGAAACT R: GCGGCATTATGCGTGATT	exon 1	307	59
P2	F: GGGGCAACCAGATTGATTCCT R: TCGGCAGCCTTGTCCAC	exon 2	197	59
P3	F: AGCCCTGCGTTCTAT R: AGGCGGAGGAGCAT	exon 10	204	55

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 (Sanguinetti *et al.*, 1994).

DNA sequencing analysis: The 14 PCR products which represented different electrophoresis patterns 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 using DNAMAN Software.

Statistical analysis: Genotypic and allelic frequencies and Hardy-Weinberg equilibriums were estimated. The statistical software SPSS (Version 13.0) was used to carry out the statistical analysis. The following model was used to analyze the association of different genotypes with twinning trait:

$$Y_{ik} = \mu + Age_i + Marker_k + e_{ik}$$

Where:

Y_{ik} = Twinning trait measured on each of ikth sheep
μ = Overall population mean
Age_i = Type of the ith age
$Marker_k$ = The fixed effect associated with kth genotype
e_{ik} = Random error

And meanwhile researchers made a χ^2-test for independence in polymorphic loci of the genotype distribution in single-twin groups (Zhang *et al.*, 2007).

RESULTS AND DISCUSSION

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. The PCR products which represented three genotypes were sequenced in both directions in ABI PRISM 377 DNA sequencer and compared to cattle *RXRG* gene sequence access No.: NC_007301. The results showed that the P2 fragment of sheep and cattle reported have three loci: 32, 125 and 146 bp, the bases of the three loci were G, T, A, respectively in sheep sequence while mutations at the cow were C, C, G. Sequence comparison revealed there were two SNPs including: g.131A>G and g.32G>A. The g.131A>G SNP accordingly contributed to AA and AB genotypes, the g.32G>A SNP accordingly contributed to BB and AB genotypes. Polymorphisms were not found in the P1and P3 fragments by SSCP analysis (Fig. 2).

The genotype frequency and gene frequency of *RXRG* gene in China Merino are shown in (Table 2). The B genotype is the predominant allele in all populations. In China Merino which produce one offspring per year, AB genotype frequency is slightly higher than BB and AA genotype frequency is the lowest. But in China twinning Merino, BB genotype frequency is the highest, AB genotype frequency is a little low and AA genotype frequency is the lowest among this populations. In Kazak sheep populations, BB genotype frequency is higher than AB and AA and the frequency differences between BB and AB are very small and AA genotype has the lowest frequences. In Hu sheep populations there are two genotyes: BB and AB, BB has the higher genotype frequency. Allele B is the predominant gene in any one of four sheep populations.

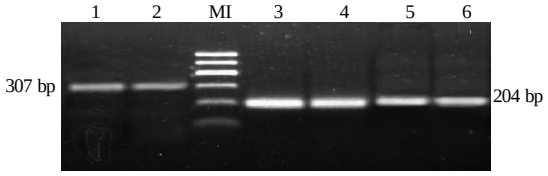

Fig. 1: PCR products of 3 pairs of primers; 1, 2: PCR products of P1; 3, 4: PCR products of P2; 5, 6: PCR products of P3; MI: SD002 marker

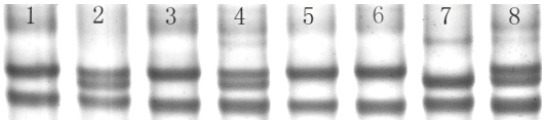

Fig. 2: SSCP analysis of PCR amplification using P2; 7: AA genotype; 2, 4, 8: AB genotype; 1, 3, 5, 6: BB genotype

Different RXRG genotypes and China Merino sheep litter size's results can be seen in Table 3. In P2 locus, the litter size of AA genotype Chinese Merino sheep is 0.22 more than AB genotype only ($p<0.05$), BB genotype Chinese Merino sheep litter size is more than 0.15 only AB genotype ($p<0.05$). The AA and BB genotypes produce more twins than the AB genotype but the AA genotype is less statistically significant. Researchers also conducted a χ^2-test for independence, China Merino genotypes in a single group composed of twin significantly different ($\chi^2 = 8.986$, $p = 0.011$) (Table 4).

In this study, genetic characteristics of single nucleotide mutation were analyed in the exon 2 of RXRG. The results revealed that there are three nucleotides differences between the amplification fragment of P2 sheep and corresponded bovine sequence reported. In experimental sheep, the bases of the three mutation sites were G, T, A, respectively while they were C, C, G in cattle. AA-type had an AG mutation in 131 bp, BB type had a GA mutation in 32 bp (Fig. 3) but such mutations were not cause amino acid changes that just indicated that these mutations were not correlated with protein expression and therefore these mutations had not influenced gene translation, bue maybe showed some subtle impact in gene duplication and transcription just because of this subtle influence, litter size had changed accordingly (Zeng *et al*., 2011). Association analysis between genotype effect and twinning trait in China Merino found that P2 mutation rate was higher which showed that this locus fit hardy weinberg equilibrium, besides there were significant differences at genotype distribution among single and twin groups ($p<0.05$), indicated that genotypes have a greater impact on the phenomenon of sheep twins. Thus, researchers can speculate that RXRG may be

Table 2: The genotype frequency and gene frequency of *RXRG* gene in China Merino

		RXRG2 primers				
		Genotype frequency			Allele frequency	
Groups	No.	AA	AB	BB	A	B
China Monotocous Merino	178	0.084 (15)	0.494 (88)	0.422 (75)	0.331	0.669
China twinning Merino	58	0.155 (9)	0.276 (16)	0.569 (33)	0.293	0.707
Kazak sheep	40	0.150 (6)	0.400 (16)	0.450 (18)	0.350	0.650
Hu sheep	37	0.000 (0)	0.270 (10)	0.730 (27)	0.135	0.865

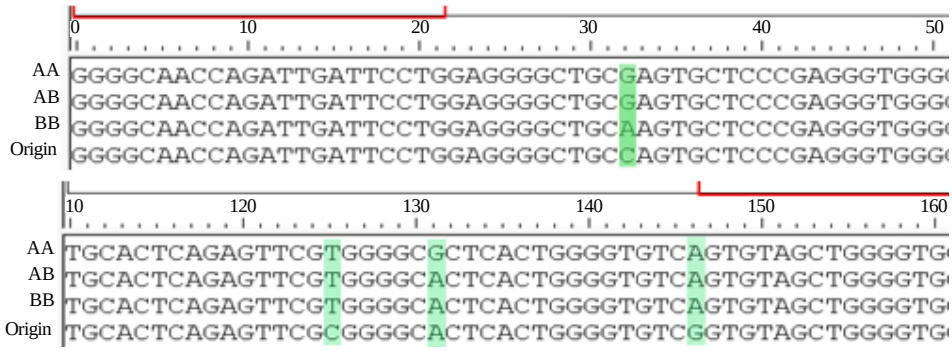

Fig. 3: Nucleotide sequences comparision of AA, AB and BB genotypes of P2

Table 3: Data of Heterozygosities (H), effective Number of alleles (Ne), Polymorphism Information Contents (PIC) and χ^2-test

Groups	PIC	H	Ne	χ^2-test
China Monotocous Merino	0.345	0.505	1.796	2.264 (p>0.05)
China Twinning Merino	0.328	0.724	1.708	6.532 (p<0.05)
Kazak sheep	0.336	0.643	1.747	3.436 (p>0.05)
Hu sheep	0.206	0.730	1.305	0.804 (p>0.05)

Table 4: Gene frequency of *RXRG* gene in different group by χ^2-test

Sheeps	χ^2-values
China Monotocous Merino and China twinning merino	8.986 (p<0.05)
China Monotocous Merino and Kazak sheep	2.138 (p>0.05)
China Monotocous Merino and Hu sheep	12.634 (p<0.05)
China twinning Merino and Kazak sheep	1.765 (p>0.05)
China twinning Merino and Hu sheep	6.668 (p<0.05)
Kazak sheep and Hu sheep	9.820 (p<0.05)

Table 5: Correlation analysis between different genotypes of RXRG

Gene locus	Breed character	Genotypes	Mean±SE
RXRG2	Lambing number	AA (24)	1.375±0.087[a]
		AB (104)	1.154±0.042[b]
		BB (108)	1.306±0.041[a]

Table 6: Correlation analysis between different genotypes of RXRG

Gene locus	Breed character	Genotypes	Mean±SE
RXRG	Birth weight	AA (15)	3.8455±0.273[a]
		AB (88)	4.3024±0.535[b]
		BB (75)	3.9133±0.345[a]

the gene which affects China Merino sheep twinning trait. (Table 5). Although, researchers did not analyze every site of *RXRG* gene in this study, researchers choose different species of sheep to study the genotype frequency and gene frequency of *RXRG* gene, like China Merino, Kazak sheep, Hu sheep. From this part, researchers could get a conclusion that allele B is the predominant gene in any one of four sheep populations. And this may provide a choice for twinning sheep.

Different *RXRG* gene type and Chinese merino single lamb group the birth weight of the correlation analysis of the results are shown in Table 6, AB genotype Chinese Merino average birth weight heavier than the AA and BB genotypes, AB genotype average birth weight is the largest with the significant difference between AA and BB genotypes ($p<0.05$) that the gene loci on Chinese Merino is of certain influence to the birth weight.

There are only a few reports about *RXRG* gene impact on animal reproductive traits. Messer *et al*. (1996) found *RARG* gene can be expressed in the critical period of pregnancy in pig and if *RARG* gene expresses then in the french large white litter which has ultra-high litter size, the average litter size increased by 0.21 pigs and in the control group increased by 0.14 L^{-1}. Huang *et al*. (2008) have found that AB genotype of *RXRG* gene in cattle would produce twins more susceptible than AA genotype and the influence of compositon of genotype in one or twin cattle were significantly different ($p = 0.0006$). Guo *et al*. (2006) found that for CC genotype of *RARG* gene, the average litter size of small tailed han are higher than the CD genetype by 0.55 ($p<0.05$). These studies show that *RAR* gene have significant impact on the fecundity of pigs and sheep and *RXRG* gene has a significant impact on fecundity of cattle. Do the *RXRG* genes also affect the sheep fecundity? In this experiment, based on results researchers studied earlier, association analysis showed that the mutation site has impact on twinning trait of sheep. Litter sizes of P2 sites of AA genotype of Chinese Merino sheep are more than AB genotype by 0.22 ($p<0.05$), litter sizes of BB genotype of Chinese Merino sheep are more than AB genotype by 0.15 ($p<0.05$), namely sheep with AA and BB genotypes easily produce twins than AB genotype but the AA genotype has little statistically significance. *RXRG* gene mutation rate in the population of Hu sheep is very low so, *RXRG* gene mutation rate in the case of other groups and its impact on other reproductive traits need further study.

CONCLUSION

Meanwhile, the results of this study will not only extend the spectrum of genetic variations of sheep *RXRG* gene but aslo benefit for reproduction traits selection.

REFERENCES

Burrage, P.S., J.T. Huntington, M.B. Sporn and C.E. Brinckerhoff, 2007. Regulation of matrix metalloproteinase gene expression by a retinoid X receptor-specific ligand. Arthritis Rheumatism, 56: 892-904.

Chambon, P., A. Zelent, M. Petkovich, C. Mendelsohn and P. Leroy *et al*., 1991. The family of retinoic acid nuclear receptors. Retinoids, 10: 10-27.

Guo, X.H., M. Chu, Z.X. Zhou, F. Li and Y.S. Cheng, 2006. Study on RARG as a candidate gene for prolif icacy of small tailed Han sheep. Chin. J. Anim. Vet. Sci., 37: 756-760.

Huang, M., S.Z. Xu, L.S. Zan, L.P. Zhang, X. Gao and J.B. Chen, 2008. Genetic variation in RXRG gene and its relationship with twinning trait in cattle. Hereditas, 30: 190-194.

Leid, M., P. Kastner and P. Chambon, 1992. Multiplicity generates diversity in the retinoic acid signalling pathways. Trends Biochem. Sci., 17: 427-433.

Leid, M., P. Kastner, B. Durand, A. Krust and P. Leroy *et al*., 1993. Retinoic acid signal transduction pathways. Ann. N. Y. Acad. Sci., 684: 19-34.

Liu, Y.F., J.F. Gao, X.L. Pan, J.S. Dai and D.Q. Li, 1997a. A study on preparation of DNA from sheep blood. J. Shihezi Univ. (Nat. Sci.), 1: 136-138.

Liu, Q., E.C. Thorland, J.A. Heit and S.S. Sommer, 1997b. Overlapping PCR for bidirectional PCR amplification of specific alleles: A rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. Genome Res., 7: 389-398.

Liu, S., J. Wang, Q. Luo and W. Li, 2005. The recent advances on leptin of dairy cattle. China Dairy Cattle, 3: 22-25.

Lohnes, D., P. Kastner, A. Dierich, M. Mark, M. LeMeur and P. Chambon, 1993. Function of retinoic acid receptor-γ in the mouse. Cell, 73: 643-658.

Messer, L., L. Wang, C. Legault and M.F. Rothschild, 1996. Mapping and investigation of candidate genes for litter size in French large white pigs. Anim. Genet., 27: 101-119.

Michaille, J.J., S. Blanchet, B. Kanzler and J.M. Garnier, 1994. Characterization of cDNAs encoding the chick retinoic acid receptor-γ2 and preferential distribution of retinoic acid receptor-γ transcripts during chick skin development. Dev. Dyn., 201: 334-343.

Mu, Y.L., M.X. Chu and S.H. Sun, 2006. PCR-SSCP analysis on Prolactin receptor gene in sheep. Acta Veterinaria et Zootechnica Sinica, 37: 956-960.

Sanguinetti, C.J., D.E. Neto and A.J. Simpson, 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques, 17: 914-921.

Van Vleck, L.D. and K.E. Gregory, 1996. Selection for twinning in cattle. J. Anim. Sci., 3: 522-528.

Wirtanen, L. and C. Seguin, 2000. Cloning of cDNAs encoding retinoic acid receptors RAR γ1, RAR γ2 and a new splicing variant, RAR γ3, from *Aambystoma mexicanum* and characterization of their expression during early development. Biochim. Biophys. Acta (BBA)-Gene Struct. Exp., 1492: 81-93.

Zeng, X.C., H.Y. Chen, B. Jia, Z.S. Zhao, W.Q. Hui, Z.B. Wang and Y.C. Du, 2011. Identification of SNPs within the sheep PROP1 gene and their effects on wool traits. Mol. Biol. Rep., 38: 2723-2728.

Zhang, D.J., 2009. Study on expression and regulation of Retinoid X receptor ßgene in porcine fibroblast. J. Zhejiang Agric. Sci., 3: 614-615.

Zhang, Y.N., E.P. Zhang, D. Wu and Y.L. Chen, 2007. Studies of the relationship between polymorphism of KAP gene and economic traits for liaoning cashmere goat. Sci. Agric. Sinica, 40: 2062-2067.