



# Expression and Polymorphism of *FSHR* Gene in Sheep with Different Fecundity

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## ABSTRACT

Follicle-stimulating hormone (FSH) is the pituitary hormone that regulates gonadal function by Hypothalamic-Pituitary-Gonadal Axis (HPGA) in vertebrates. FSHR has the function of transmitting follicle-stimulating growth biological information in animal breeding activities. In this study, we analyzed the structure of *FSHR* gene's promoter, expression regulation and the correlation between polymorphism with fecundity. The results show that it is highly possible that the promoter of *FSHR* gene is in the region of 2045-2295bp and 2038-2288bp, TATA box was also found there. The expression differences were found in oviduct during every stage of estrous cycle ( $P < 0.05$ ) between Small Tail Han (STH) sheep and Tan (T) sheep. There were significant differences in genotype distribution between STH sheep and T sheep at T1235C and AG2357-2358 CT sites ( $P < 0.01$ ). The results of haplotypes analysis indicated that H2, H7 were preponderant haplotype in high prolificacy sheep breed. The litter size of TT genotype was significantly higher than that in CC genotype at T1235C loci in STH sheep ( $P < 0.05$ ). The current study provides evidence in sheep for genetic markers that might be used in breeding program.

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## Authors' Contribution

XH did formal analysis and wrote the draft. YL provided visualization. JW curated data. RD managed software work. QL performed the experiments. WH and XW validated the results. MC did fund acquisition.

## Key words

*FSHR* gene, Polymorphism, Litter size, Haplotype analysis, Hypothalamic-Pituitary-Gonadal Axis

## INTRODUCTION

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the pituitary hormones that regulate gonadal function by Hypothalamic-Pituitary-Gonadal Axis (HPGA) in vertebrates (Pérez-Solis *et al.*, 2010). FSH and LH are also required for normal development and function of gonads in females through their cognate receptors, the FSHR and the LHR (Richards *et al.*, 1987; Heckert *et al.*, 1992; Sacchi *et al.*, 2018). By binding to its receptor, which stimulates the formation of cAMP system and activation of multiple intracellular signaling cascades that influence expression of FSH-dependent genes in the gonads (Ulloaaguirre *et al.*, 2007). The FSHR has the function of transmitting follicle-stimulating growth biological information in animal breeding activities. Its genetic mutations may enhance or weaken the function of FSH information transduction, which has a profound influence on reproductive phenotypes. These receptors belong to the superfamily of G-protein coupled receptors

that have seven transmembrane spanning domains and a large extracellular domain that interact with the  $\alpha$  and  $\beta$  subunits of the FSH, which are encoded by a single copy gene (Griswold *et al.*, 1995). It has been reported that the genetic mutations of FSHR cDNA may affect the ability of FSH signal transduction (Sprengel *et al.*, 1990). Abdennebi *et al.* (1999) found that both FSHR mRNA levels and responsiveness of viable granulosa cells to identical dose of FSH were higher in growth ovarian follicles of polytocous Romanov ewes than in monotocous Ile-de-France ewes, which demonstrated that the activity of FSH may be regulated by the expression level of FSHR (Nagirnaja *et al.*, 2010). It has also been shown that inadequacy of follicular receptors may cause infertility (De *et al.*, 2003; Zilaitiene *et al.*, 2018).

The sheep *FSHR* gene is located on chromosome 3 and contains ten exons and nine introns (Pan *et al.*, 2014). The first nine exons encode the large extracellular domain and exon 10 encodes the transmembrane and intracellular domains, and is highly conserved amongst all species examined so far. The promoter lies on 5' upstream region of sheep *FSHR* and consists of two parts: core promoter and upstream element. The core promoter is composed of many transcription regulators, such as the region of

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regulate transcription initiation site and protein factors. Absence of usual TATA and CCAAT promoter elements and the presence of multiple transcriptional initiation sites are typical promoter features of rats (Heckert *et al.*, 1992) and human (Gromoll *et al.*, 1996) *FSHR* genes. Sairam and Subbarayan (2015) found only a single major transcription start site at -163 of the 5' flanking region. More importantly, variants in *FSHR* gene were shown to be associated with prolificacy in bovine (Rahal *et al.*, 2010), sheep (Chu *et al.*, 2012) and pigs (Wu, 2012). Researchers also found that the sensitivity of ovaries to FSH and the number of women's ovulation were associated with the 10th exon mutations Asn680Ser of *FSHR* gene (De *et al.*, 2003; Andrá *et al.*, 2018).

In this study, we predicted the promoter region of *FSHR* gene and analyzed the expression in reproductive organs at different estrous cycles of STH sheep, polymorphisms and association of the ovine *FSHR* gene to fertility in sheep was also analyzed. We also investigated the relevance between genetic variations and reproduction performance in sheep populations. The study of this gene could provide theoretic basis to explore the molecular mechanism of sheep prolificacy.

## MATERIALS AND METHODS

### Animals

All the experimental procedures mentioned in the present study were approved by the Science Research Department (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China). Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS (No. IASCAAS-AE-03, 12 December 2016).

All animal tissue collection procedures were previously described in detail (Di *et al.*, 2014). Briefly, non-pregnant Tan (T) and Small Tail Han (STH) ewes that were free of non-infectious diseases were selected from their respective breed conservation farms and housed in the same farm in Ningxia Autonomous Region, China. All diets were based on alfalfa, corn silage and a combination of concentrates including corn, soymeal and bone meal. Three ewes at each reproductive stage (dioestrus, proestrus, oestrus) were arbitrarily selected and killed for hypothalamus, pituitary, ovary, uterus and oviduct collection. All samples were immediately snapfrozen in liquid nitrogen for future use. Besides, Blood samples were obtained from 43 Hu (H) sheep, 206 Small Tail Han (STH), 41 Wadi (W), 32 Tan (T) sheep in Yuhang, Zhejiang province, Jiexiang, Shandong province, Zhanhua, Shandong province and Ningxia Hui Autonomous Region, respectively.

### DNA extraction, promoters cloning and sequence analysis

Genomic DNA was extracted from whole blood by phenol-chloroform method. The predominant PCR product was purified by gel, and subsequently cloned into the pEGM-T-Easy vector (Promega) prior to sequencing.

The *FSHR* promoters sequence of sheep (GenBank No. NW\_004080166) was employed as a template. The primer pairs were designed in partial of 5' flanking region of the ovine *FSHR* gene (*FSHR*-P<sub>n</sub>-F, *FSHR*-P<sub>n</sub>-R for *FSHR*, Table I). Promoter 2.0 Prediction Server and Neural Network Promoter was used to predict the Promoters. The promoter region transcription factor binding sites were analyzed with the Prediction Promoter SCAN. The promoter region CpG islands were analyzed by CpG island and MethPrimer.

### Tissue expression analysis of the sheep *FSHR* gene

The sheep *FSHR* gene mRNA levels in different oestrus cycles in different tissues were detected, the tissues were from hypothalamus, pituitary, ovary, uterus, oviduct of three 4-years-old STH ewes and T ewes. The total RNA of each tissue was extracted and subsequently reverse-transcribed into cDNA. The specific primer (*FSHR*-expression-F, *FSHR*-expression-R for *FSHR*, Table I) of sheep *FSHR* was used to amplify the products of 108bp. The PCR reaction was performed under 95 °C for 10 min, followed by 95 °C 15s, 60 °C 60s for 45 cycles. Actin was used as an internal control gene. qPCR was performed on the LightCycler 480II (Roche, Basel, Sweden) using SYBR Green Real time PCR Master Mix (TOYOBOCO, LTD, Japan) as the readout. The 2<sup>-ΔΔCt</sup> method was used to analyze the data.

### SNP identification and association analysis

The SNPs of the sheep *FSHR* gene was identified by cloning and sequencing the PCR products which were amplified using the eight DNA mixed sample of four sheep breeds respectively. Three pairs of primers were designed according to coding sequence of ovine *FSHR* gene (NC\_019460) for the PCR-RFLP. Two exons of *FSHR* gene were amplified, in which primers *FSHR*-SNP1 were amplified exon 1 and other two pairs of primers (*FSHR*-SNP2, *FSHR*-SNP3) for exon 10, respectively. These primers were synthesized by Shanghai Invitrogen Biotechnology Limited Corporation (Shanghai, P.R. China). Primer sequence and product size were listed in Table 1. The DNA used in this process was extracted from the blood of 322 ewes from H Sheep (n=43), STH Sheep (n=206), W Sheep (n=41) and T Sheep (n=32). The PCR for RFLP was performed in a volume of 10 μL consisting of 4 μL of PCR production, 0.5 μL of XmnI/ BsmI, 1 μL 10×buffer and 4.5 μL ddH<sub>2</sub>O. The litter size was recorded.

**Table I. Primer pairs designed for the sheep *FSHR* and *Actin* genes.**

Primer name	Primer Sequences (5'→3')	Product size (bp)	Annealing temperature (°C)
<i>FSHR</i> -P1-F	GCTATTTGTCTGGAAGTGACCGATAA	1068	57
<i>FSHR</i> -P1-R	GAGAGTACAGCACCCAAGAACGAATG		
<i>FSHR</i> -P2-F	GGGATTTGTGGGGTGGGGGTAT	1636	57
<i>FSHR</i> -P2-R	AGATTGCCCTTGCCTTTTGGATG		
<i>FSHR</i> -P3-F	GCGGCTGCTGCATTACCTAAA	1510	57
<i>FSHR</i> -P3-R	TTACAGTTCGACCGCATCCCT		
<i>FSHR</i> -expression-F	CCCATCTTTGGCATCAGCAGC	108	60
<i>FSHR</i> -expression-R	ACATTGAGCAACAAGGAGGGACATAA		
<i>Actin</i> -F	GCAAAGACCTCTACGCCAACACG	116	60
<i>Actin</i> -R	CTTGATCTTCATCGTGGTGGTG		
<i>FSHR</i> -SNP1-F	GCTATTTGTCTGGAAGTGACCGATAA	627	58
<i>FSHR</i> -SNP1-R	GAGAGTACAGCACCCAAGAACGAATG		
<i>FSHR</i> -SNP2-F	ATGGGGTATGATATTCTCAGAGTCTTGAAA	112	54
<i>FSHR</i> -SNP2-R	GGACTGTGAGTTTATACTGGCTGGTG		
<i>FSHR</i> -SNP3-F	AACTCCTGTGCCAACCC	501	52
<i>FSHR</i> -SNP3-R	CAAGAGCACTTTGAACGC		

**Table II. Predicted transcriptional binding sites of sheep *FSHR* promoter by Promoter SCAN.**

Breeds	Items	+/-	Site	Bytes	
Small Tail Han sheep	(SRF)	+	2153	2.868	
	(SRF)	-	2162	3.155	
	AP-2	+	2200	1.108	
	PuF	-	2206	1.391	
	JCV_repeat-ed_sequenc	-	2206	1.658	
	INF.1	+	2258	1.044	
	GCF	+	2289	2.361	
	UCE.2	-	2294	1.216	
	Tan sheep	(SRF)	+	2150	2.868
		(SRF)	-	2159	3.155
AP-2		+	2197	1.108	
JCV_repeat-ed_sequenc		-	2203	1.658	
PuF		-	2203	1.391	
UCE.2		+	2259	1.278	
UCE.2		-	2262	1.216	
GCF		+	2286	2.361	

The PROC GLM procedure in SAS software package was applied to analyze the association among the haploid type, genotypes and trait. The linear model with the fixed effects was:  $y_{ijklm} = \mu + S_i + LS_j + P_k + G_l + e_{ijklm}$ , where  $y_{ijklm}$  is the phenotypic value of litter size;  $\mu$  is the population mean;

$S_i$  is the fixed effect of the  $i$ th sire ( $i = 1, 2, 3, 4, 5$ );  $LS_j$  is the fixed effect of the  $j$ th lambing season ( $j = 1, 2, 3, 4$ );  $P_k$  is the fixed effect of the  $k$ th parity ( $k = 1, 2, 3$ );  $G_l$  is the fixed effect of the  $l$ th genotype ( $l = 1, 2, 3$ ), and  $e_{ijklm}$  is the random residual effect of each observation. Analysis was performed using the general linear model procedure of SAS (Ver 9.0) (SAS Institute Inc, Cary, NC, USA). Mean separation procedures were conducted using a least significant difference test.

#### Sequence analysis of sheep *FSHR*

The 5' upstream region sequences in length 2582bp were obtained from STH and T sheep two breeds, which sequences included the first exon and promoter of *FSHR* gene. The results of bioinformatics analysis shown it is highly possible that the promoters of *FSHR* gene for STH sheep and T sheep were in region of 2045-2295bp and 2038-2288bp, which the TATA box was found in these regions in both two breeds (Table II). INF. 1 transcriptional binding site was only found in Small Tail Han sheep.

#### Analysis of expression

qPCR was used to investigate the general tissue distributions of the *FSHR* gene in STH sheep and T sheep, respectively. As shown in Fig. 1, both breeds the *FSHR* gene were expressed in the hypothalamus, pituitary, ovary, uterus and oviduct, but the *FSHR* gene expression patterns of the STH sheep and T sheep were different. For STH sheep, *FSHR* gene expression was significantly higher than other tissues in pituitary at the oestrus ( $P < 0.05$ ) (Fig. 1A); for T sheep, *FSHR* gene expression was significantly

higher than other tissues in hypothalamus and oviduct at the proestrus ( $P<0.05$ ) (Fig. 1B). The relative abundance of transcripts of the *FSHR* gene in the hypothalamus, pituitary, ovary, uterus and oviduct of the 2 breeds in different estrus cycle are shown in Fig. 2. At the dioestrus the STH sheep *FSHR* gene expression was significantly higher than T sheep in oviduct ( $P<0.05$ ) (Fig. 2A); at the proestrus the Small Tail Han sheep *FSHR* gene expression was significantly higher than Tan sheep in hypothalamus and oviduct ( $P<0.05$ ), but almost no expression in the uterus (Fig. 2B); at the oestrus the Small Tail Han sheep *FSHR* gene expression was significantly higher than Tan sheep in pituitary and oviduct ( $P<0.05$ ) (Fig. 2C).

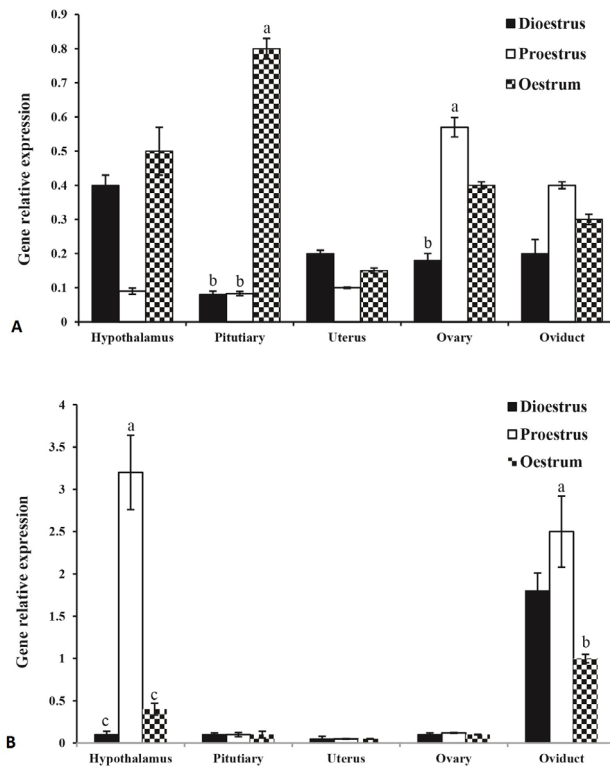


Fig. 1. *FSHR* gene expression difference in estrous cycle in STH sheep(A) and T sheep (B).

#### SNP scanning of sheep *FSHR* gene

Three mutations, g.149A>G, g.1235T>C, and g.2357-2358AG>CT, were discovered in ovine *FSHR* gene. The g.149A>G mutation located in exon1 and the other two mutations were both found to locate in exon 10. Three pairs of primers (*FSHR*-SNP1-F, *FSHR*-SNP1-R; *FSHR*-SNP2-F, *FSHR*-SNP2-R and *FSHR*-SNP3-F, *FSHR*-SNP3-R) in Table I were designed to amplify 627, 112 and 501bp fragments, respectively.

These substitutions described above were the nonsense mutations. The g.149A>G mutation was detected by PCR-RFLP using the BsmI restriction enzyme, which generated three fragments: the 627bp band representing the allele A, and the 416bp and 211bp band representing allele G (Fig. 3A). The g.1235T>C mutation was detected by the XmnI restriction enzyme, which yielded two fragments: the 112bp band representing the allele T, and 96bp band for allele C (Fig. 3B). Using Haploview software linkage analysis of these two sites, results show that the  $r^2=1$ . So, in A2357C and G2358T two loci are completely chain, mutations can be seen as one site. The g.2357-2358 AG>CT mutation was sequencing analysis of different genotypes (Fig. 3C).

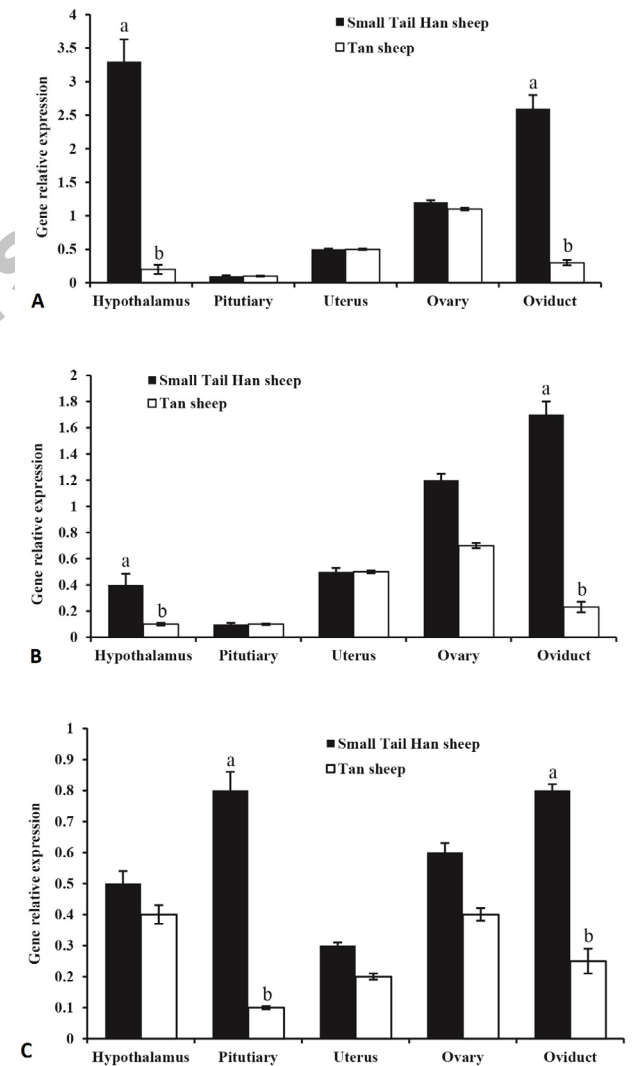


Fig. 2. Expression of *FSHR* at Dioestrus (A), Proestrus (B) and Oestrus (C).



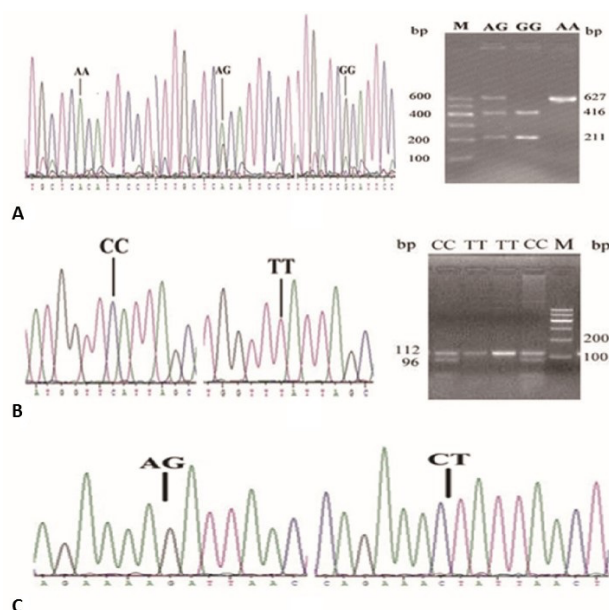


Fig. 3. PCR-RFLP results of different genotypes of the sheep *FSHR* genes. The genotypes are marked on the top of the lanes. A: PCR products digested by enzyme *BsmI* showed different genotypes of *FSHR* g.149A>G. B: PCR products digested by enzyme *XmnI* showed different genotypes of *FSHR* g.1235T>C. C: sequencing analysis of different genotypes of *FSHR* g.2357-2358 AG>CT.

#### Allele and genotype frequencies of *FSHR* gene in four sheep breeds

Allele and genotype frequencies of the g.149A>G, g.1235T>C, and g.2357-2358 AG>CT, mutations of the *FSHR* gene in four sheep breeds were presented in Table III. For g.149A>G, polymorphisms in exon 1 of *FSHR* gene were found in four sheep breeds (AA, AG and GG), the frequency of alleles A and G were similar. Regarding g.1235T>C, polymorphisms in exon 10 of *FSHR* gene were also found in four sheep breeds (TT and CC), in high fecundity sheep breeds for STH sheep and W sheep, the T and TT was dominant allele and superior genotype, respectively. Regarding g. 2357-2358 AG>CT, polymorphisms in exon 10 of *FSHR* gene were found in four sheep breeds (AG and CT), the genotype AG was preponderant genotype in H and T sheep, the genotype CT was preponderant genotype in STH sheep and W sheep.

#### Haplotype analysis of *FSHR* gene mutations site

Haplotypes and Haplotype frequencies of the three mutations of the *FSHR* gene in four sheep breeds were presented in Table IV and Table V. Haplotype frequency

analysis shows in high fecundity sheep breeds for H sheep, STH sheep and W sheep the H2 and H7 was dominant haplotypes, respectively. in low fecundity sheep breeds for T sheep the H1 and H7 was dominant haplotypes. Regarding haplotype combination frequency, for H sheep, STH sheep, W sheep and T sheep the H2/H3, H2/H7, H2/H5 and H1/H8 was dominant haplotypes, respectively.

Table III. Allele and genotype frequencies of *FSHR* in four sheep breeds

SNPs	Breeds	Hu sheep	Small Tail Han sheep	Wadi sheep	Tan sheep	
T1235C	No.	43	206	41	32	
	Allele frequencies	TT	0.72(31)	0.78(161)	0.71(29)	0.47(15)
		TC	0(0)	0(0)	0(0)	0(0)
	Genotype frequencies	CC	0.28(12)	0.22(45)	0.29(12)	0.53(17)
		T	0.72	0.78	0.71	0.47
	C	0.28	0.22	0.29	0.53	
H-W test	$\chi^2$	43.0***	206.0***	41.0***	32.0***	
AG2357-2358CT	No.	43	206	41	32	
	Genotype frequencies	AG	0.56(24)	0.33(68)	0.41(17)	0.62(20)
		CT	0.44(19)	0.67(138)	0.59(24)	0.38(12)
A149G	No.	43	206	41	32	
	Genotype frequencies	AA	0.21(9)	0.43(88)	0.34(14)	0.34(11)
		AG	0.42(18)	0.22(45)	0.24(10)	0.28(9)
		GG	0.37(16)	0.35(73)	0.42(17)	0.38(12)
	Allele frequencies	A	0.42	0.54	0.46	0.48
		G	0.58	0.46	0.54	0.52
H-W test	$\chi^2$	0.84	64.78***	26.02***	6.11*	

Note: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

Table IV. Haplotype frequencies of in *FSHR* gene in four sheep breeds.

Haplotype	Hu sheep	Small Tail Han sheep	Wadi sheep	Tan sheep
H1 TAGA	0.151(13)	0.097(40)	0.195(16)	0.297(19)
H2 TAGG	0.268(23)	0.311(128)	0.195(16)	0.078(5)
H3 TCTA	0.174(15)	0.092(38)	0.098(8)	0.078(5)
H4 CAGA	0.058(5)	0.170(70)		
H5 CAGG	0.081(7)	0.078(32)	0.195(16)	0.109(7)
H6 CCTA	0.023(2)		0.073(6)	0.078(5)
H7 CCTG	0.244(21)	0.252(104)	0.244(20)	0.266(17)
H8 TCTG				0.094(6)

**Table V. Diplotype frequencies of *FSHR* gene in four sheep breeds.**

Diplotype	Hu sheep	Small Tail Han sheep	Wadi sheep	Tan sheep
H1/H1	0.047(2)	0.058(12)	0.049(2)	0.094(3)
H1/H2	0.023(1)	0.068(14)	0.073(3)	0.031(1)
H1/H5	0.047(2)		0.122(5)	0.031(1)
H1/H6	0.047(2)		0.098(4)	0.125(4)
H1/H7	0.093(4)	0.010(2)		0.031(1)
H1/H8				0.188(6)
H2/H3	0.302(13)	0.087(18)	0.024(1)	
H2/H4		0.214(44)		
H2/H5	0.093(4)	0.117(24)	0.171(7)	0.063(2)
H2/H7	0.116(5)	0.136(28)	0.122(5)	0.063(2)
H3/H4		0.010(2)		
H3/H7	0.047(2)	0.087(18)	0.171(7)	0.156(5)
H4/H7	0.116(5)	0.117(24)		
H5/H6			0.049(2)	0.031(1)
H5/H7	0.023(1)	0.039(8)	0.049(2)	0.094(3)
H7/H7	0.047(2)	0.058(12)	0.073(3)	0.094(3)

**Table VI. Least squares mean and standard error for litter size of different genotypes of the three SNP mutation loci in *FSHR* gene in Small Tail Han sheep.**

SNPs	Genotype	No.	litter size
g.1235T>C	TT	161	2.37 <sup>a</sup> ±0.10
	CC	45	1.95 <sup>b</sup> ±0.18
g.2357-2358AG>CT	AG	68	2.23 <sup>a</sup> ±0.16
	CT	138	2.30 <sup>a</sup> ±0.11
g.149A>G	AA	88	2.35 <sup>a</sup> ±0.12
	AG	45	2.27 <sup>a</sup> ±0.19
	GG	73	2.19 <sup>a</sup> ±0.13

Note: Least squares means with the same superscript for the same polymorphic locus have no significant difference ( $P>0.05$ ). Least squares means with the different superscripts for the same polymorphic locus differ significantly ( $P<0.05$ ).

#### *Influence of fixed effects on litter size in STH sheep*

Litter size in STH sheep was significantly influenced by sire, lambing season, parity, and *FSHR* genotype. The least squares mean (LSM) and standard error for litter size of different genotypes of *FSHR* gene three mutations in STH sheep were given in Table VI. As shown in Table VI, for g.1235T>C mutation, STH sheep ewes with genotype TT had 0.42 ( $P<0.05$ ) lambs more than those with genotype CC. The results indicated that allele T was significantly

correlated with high prolificacy. For g.149A>G and g.2357-2358AG>CT, the differences of the LSM for litter size were not significant ( $P>0.05$ ). No significant were found between the other haplotype combinations and litter size in STH sheep, except of haplotype combination H5/H7 and H7/H7 (Table VII).

**Table VII. Least squares mean and standard error for litter size of different diplotype of the three SNP mutation loci in *FSHR* gene in Small Tail Han sheep.**

Diplotype	No.	litter size
H1/H1	12	2.38 <sup>a</sup> ±0.14
H1/H2	14	2.40 <sup>a</sup> ±0.14
H1/H7	2	2.27 <sup>abc</sup> ±0.16
H2/H3	18	2.42 <sup>a</sup> ±0.12
H2/H4	44	2.35 <sup>a</sup> ±0.09
H2/H5	24	2.24 <sup>abc</sup> ±0.11
H2/H7	28	2.28 <sup>ab</sup> ±0.10
H3/H4	2	2.39 <sup>abc</sup> ±0.16
H3/H7	18	2.30 <sup>ab</sup> ±0.12
H4/H7	24	2.23 <sup>abc</sup> ±0.11
H5/H7	8	1.85 <sup>bc</sup> ±0.15
H7/H7	12	1.95 <sup>b</sup> ±0.14

Note: Least squares means with the same superscript for the same polymorphic locus have no significant difference ( $P>0.05$ ). Least squares means with the different superscripts for the same polymorphic locus differ significantly ( $P<0.05$ ).

## DISCUSSION

According to the software analysis the 5' upstream region sequences of *FSHR* gene found the exon 1, and combination with CpG inland promoter regions. This method does not contain the CpG island gene forecast accuracy is low (Davuluri *et al.*, 2001). Another method is based on the transcription in the promoter region of the signals, such as CCAAT box and the TATA box, and combined with the recognition of these transcription signal to determine promoter regions, also this method there is a shortage, is easy to form a false positives, reduce the accuracy of the prediction (Abdennebi *et al.*, 1999). In this study, it is highly possible that the promoters of *FSHR* gene for STH sheep and T sheep were in region of 2045-2295bp and 2038-2288bp, which the TATA box was found in these regions in both two breeds. INF.1 transcriptional binding site was only found in STH sheep.

*FSHR* mRNA level has been shown to be correlated to the follicular development phases in cattle (Xu *et al.*,

1995; Madrid Gaviria *et al.*, 2015; Kumar, 2017), however, *FSHR* expression level is not only related with the follicular phases, but also had significant difference between breeds (Abdennebi *et al.*, 1999). The tissue expression in our study showed that *FSHR* gene were expressed in the hypothalamus, pituitary, ovary, uterus and oviduct, but the *FSHR* gene expression patterns of the STH sheep and T sheep were different. Our results of qPCR showed that *FSHR* gene expression was significantly higher than other tissues in pituitary at the oestrus in STH sheep ( $P<0.05$ ); while *FSHR* gene expression was significantly higher than other tissues in hypothalamus and oviduct at the proestrus in T sheep ( $P<0.05$ ). The result was generally consistent with the previously reported about *FSHR* gene expression in sheep ovary (Abdennebi *et al.*, 1999; Wei *et al.*, 2017; Patel *et al.*, 2018). During male fetal, neonatal and pubertal periods, *FSH* stimulates proliferation of testicular Sertoli cells determining spermatogenic capacity of adult testes, and it contributes to normal spermatogenesis and spermatogonial survival and sperm release in adulthood (Ruwanpura *et al.*, 2010). It mediates *FSHR* through its biological functions (Chu *et al.*, 2012). The level of *FSH* is controlled by *FSHR*, and aberrant *FSHR* affects ovary and folliculogenesis (Fauser and Van Heusden, 1997). The well characterized G protein-coupled form of the *FSHR*, alternatively spliced variants of the *FSHR* may participate in follicular dynamics during follicular waves of the sheep estrous cycle (Sullivan *et al.*, 2013). From our result we could deduce that *FSHR* may take part in the process of reproductive between the different sheep breeds (high and low sheep breed).

Three synonymous mutation, g.149A>G, g.1235T>C, and g.2357-2358AG>CT were discovered in sheep *FSHR* gene. And for the g.2357-2358AG>CT, the genotype AG was preponderant genotype in H and T sheep, the genotype CT was preponderant genotype in STH sheep and W sheep. For the g. 149A>G, the frequency of alleles A and G were similar in four sheep breeds. Regarding g. 1235T>C, in high fecundity sheep breeds for STH sheep and W sheep, the T and TT was dominant allele and superior genotype, respectively. And the association analysis of sheep *FSHR* showed that the novel SNP g.1235T>C mutation detected in *FSHR* gene had significant effects ( $P<0.05$ ) on trait of litter size. All the phenotype values of the litter size in the animals with the TT genotype were evidently higher than those with the CC genotype. This indicated that allele T contributed higher phenotype values than allele C. The TT Genotype can provide, on average, 0.42 more litter size than genotype CC. Additionally, Chu *et al.* also found four mutations in the 5' UTR of sheep *FSHR* gene, and the distribution of SNPs is evidently different in sheep breeds, and it was assumed that the *FSHR* gene may affect

the litter size of sheep significantly (Chu *et al.*, 2012). The mutation (g. 47C>T) in *FSHR* were found in the above two sheep breeds with a total number of 1630 individuals, The single marker association analysis showed that the three mutations were significantly associated with litter size (Sullivan *et al.*, 2013; Wei *et al.*, 2013; Pan *et al.*, 2014; Wang *et al.*, 2015). Taken these together, sheep *FSHR* gene can be used as a molecular marker in improving the litter size of sheep.

## CONCLUSION

Our results indicated the relation of *FSHR* to reproduction in sheep and the *FSHR* gene could be used as a candidate gene for improving the litter size in industrial sheep breeding. Subsequently, we will conduct further study on our results in a larger sheep population.

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### Statement of conflicts of interest

The authors declare that they have no competing interests.

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