Pakistan J. Zool., vol. 54(2), pp 667-675, 2022 DOI: https://dx.doi.org/10.17582/journal.pjz/20190215010208

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

Xiaoyun He[1], Yongfu La[2], Jinxin Wang[1], Ran Di[1], Qiuyue Liu[1], Xiangyu Wang[1], Wenping Hu[1] and Mingxing Chu[1]*

[1]*Key Laboratory of Animal Genetics, Breeding and Reproduction of Ministry of Agriculture, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China*
[2]*College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730070, China.*

Xiaoyun He and Yongfu La contributed equally to this article.

Article Information
Received 15 February 2019
Revised 29 April 2019
Accepted 14 May 2019
Available online 23 April 2021 (early access)
Published 10 January 2022

Authors' Contribution
XH did formal analysis and wrote the draft. YL provided visualization. JW curated data. RD managed software work. QL performed the esperiments. WH and XW validaed the results. MC did fund acquisition.

Key words
***FSHR* gene, Polymorphism, Litter size, Hyplotype analysis, Hypothalamic-Pituitary-Gonadal Axis**

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.

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 α and β 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

* Corresponding author: mxchu@263.net
0030-9923/2022/0002-0667 $ 9.00/0

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; Andrã *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, oestrum) 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, Jiaxiang, 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_n-F, *FSHR*-P_n-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 ^{0}C for 10 min, followed by 95 ^{0}C 15s,60 ^{0}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 $2^{-\Delta\Delta Ct}$ 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 RFRPwas performed in a volume of 10μL consisting of 4 μL of PCR production, 0.5 μL of XmnI/ BsmI, 1 μL 10×buffer and 4.5 μL ddH2O 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 (^{0}C)
FSHR-P1-F *FSHR*-P1-R	GCTATTTGTCTGGAAGTGACCGATAA GAGAGTACAGCACCCAAGAACGAATG	1068	57
FSHR-P2-F *FSHR*-P2-R	GGGATTTGTGGGGTGGGGGTAT AGATTGCCCTTGCCTTTTGGATG	1636	57
FSHR-P3-F *FSHR*-P3-R	GCGGCTGCTGCATTACCTAAA TTACAGTTCGACCGCATCCCT	1510	57
FSHR-expression-F *FSHR*-expression-R	CCCATCTTTGGCATCAGCAGC ACATTGAGCACAAGGAGGGACATAA	108	60
Actin-F *Actin*-R	GCAAAGACCTCTACGCCAACACG CTTGATCTTCATCGTGCTGGGTG	116	60
FSHR-SNP1-F *FSHR*-SNP1-R	GCTATTTGTCTGGAAGTGACCGATAA GAGAGTACAGCACCCAAGAACGAATG	627	58
FSHR-SNP2-F *FSHR*-SNP2-R	ATGGGGTATGATATTCTCAGAGTCTTGAAA GGACTGTGAGTTTATACTGGCTGGTG	112	54
FSHR-SNP3-F *FSHR*-SNP3-R	AACTCCTGTGCCAACCC CAAGAGCACTTTGAACGC	501	52

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_repeated_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_repeated_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; μ is the population mean; S_i is the fixed effect of the ith sire (i = 1,2, 3, 4, 5); LS_j is the fixed effect of the jth lambing season (j = 1, 2, 3, 4); P_k is the fixed effect of the kth parity (k = 1, 2, 3); G_l is the fixed effect of the lth 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 oestrum ($P<0.05$) (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 dioestrum the STH sheep *FSHR* gene expression was significantly higher than T sheep in oviduct(P<0.05) (Fig. 2A); at the proestrum 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 oestrum the Small Tail Han sheep *FSHR* gene expression was significantly higher than Tan sheep in pituitary and oviduct (P<0.05) (Fig. 2C).

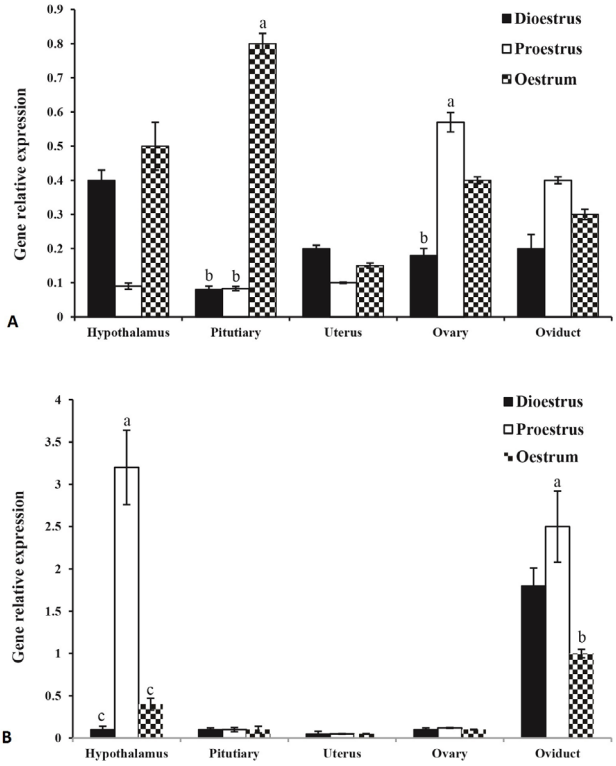

Fig. 1. *FSHR* gene expression difference in estrous cycle in STH sheep(A) and T sheep (B).

SNP scanning of sheep FSHR *gene*

Tree 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. Tree 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 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).

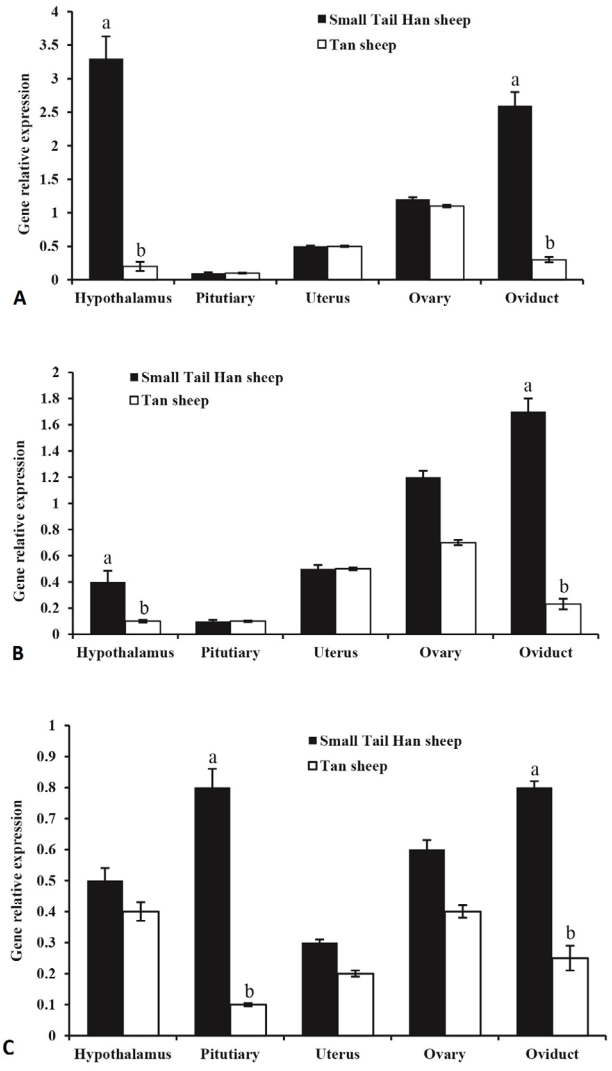

Fig. 2. Expression of FSHR at Dioestrus (A),Proestrus (B) and Oestrum(C).

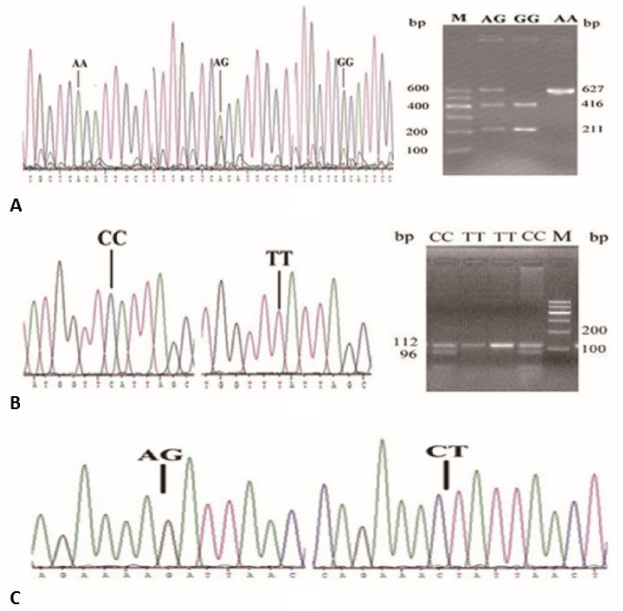

Fig. 3. PCR-RFLP results of different genotypes of the sheep *FSHR* genes. The genotypes are marked on the top of the lanes. A: PCR products digested by enzyme *BsmI* showed different genotypes of *FSHR* g.149A>G. B: PCR products digested by enzyme *XmnI* showed different genotypes of *FSHR* g.1235T>C. 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)
		CC	0.28(12)	0.22(45)	0.29(12)	0.53(17)
	Genotype frequencies	T	0.72	0.78	0.71	0.47
		C	0.28	0.22	0.29	0.53
	H-W test	χ^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)	0.4217)	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	χ^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^{a}±0.10
	CC	45	1.95^{b}±0.18
g.2357-2358AG>CT	AG	68	2.23^{a}±0.16
	CT	138	2.30^{a}±0.11
g.149A>G	AA	88	2.35^{a}±0.12
	AG	45	2.27^{a}±0.19
	GG	73	2.19^{a}±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^{a}±0.14
H1/H2	14	2.40^{a}±0.14
H1/H7	2	2.27^{abc}±0.16
H2/H3	18	2.42^{a}±0.12
H2/H4	44	2.35^{a}±0.09
H2/H5	24	2.24^{abc}±0.11
H2/H7	28	2.28^{ab}±0.10
H3/H4	2	2.39^{abc}±0.16
H3/H7	18	2.30^{ab}±0.12
H4/H7	24	2.23^{abc}±0.11
H5/H7	8	1.85^{bc}±0.15
H7/H7	12	1.95^{b}±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.1transcriptional 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 oestrum 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.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (Grant No. 31472078 and 31772580), the Earmarked Fund for China Agriculture Research System (Grant No. CARS-38), Agricultural Science and Technology Innovation Program of China (Grant No. ASTIP-IAS13), Central Public-interest Scientific Institution Basal Research Fund (Grant No. Y2017JC24), China Agricultural Scientific Research Outstanding Talents and Their Innovative Teams Program, China High-level Talents Special Support Plan Scientific and Technological Innovation Leading Talents Program (Grant No. W02020274), Tianjin Agricultural Science and Technology Achievements Transformation and Popularization Program (Grant No. 201704020).

Statement of conflicts of interest

The authors declare that they have no competing interests.

REFERENCES

Abdennebi, L., Monget, P., Pisselet, C., Remy, J.J., Salesse, R. and Monniaux, D., 1999. Comparative expression of luteinizing hormone and follicle-stimulating hormone receptors in ovarian follicles from high and low prolific sheep breeds. *Biol. Reprod*., **60**: 845-854. https://doi.org/10.1095/biolreprod60.4.845

Andrã, G.M., Martins, T.C., Pedruzzi, I.N., Rfm,F., Oliveira, R., Christofolini, D.M., Bianco, B. and Barbosa, C.P., 2018. The impact of *FSHR* gene polymorphisms Ala307Thr and Asn680Ser in the

endometriosis development. *DNA Cell. Biol*., **37**: 584-591. https://doi.org/10.1089/dna.2017.4093

Chu, M.X., Guo, X.H., Feng, C.J., Li, Y., Huang, D.W., Feng, T., Cao, G.L., Fang, L., Di, R. and Tang, Q.Q., 2012. Polymorphism of 5' regulatory region of ovine *FSHR* gene and its association with litter size in small tail Han sheep. *Mol. Biol. Rep*., **39**: 3721-3725. https://doi.org/10.1007/s11033-011-1147-x

Davuluri, R.V., Grosse, I. and Zhang, M.Q., 2001. Computational identification of promoters and first exons in the human genome. *Nat. Genet*., **29**: 412-417. https://doi.org/10.1038/ng780

De, C.F., Ruiz, R., Montoro, L., Pérezhernández, D., Sánchezcasas, P.E., Real, L.M. and Ruiz, A., 2003. Role of follicle-stimulating hormone receptor Ser680Asn polymorphism in the efficacy of follicle-stimulating hormone. *Fertil. Steril*., **80**: 571-576. https://doi.org/10.1016/S0015-0282(03)00795-7

Di, R., He, J.N., Song, S.H., Tian, D.M., Liu, Q.Y., Liang, X.J., Ma, Q., Sun, M., Wang, J.D., Zhao, W.M., Cao, G.L., Wang, J.X., Yang, Z.M., Ge, Y. and Chu, M.X., 2014. Characterization and comparative profiling of ovarian microRNAs during ovine anestrus and the breeding season. *BMC Genom.,* **15**: 899. http://doi.org/1471-2164-15-899

Fauser, B.C. and Van Heusden, A.M., 1997. Manipulation of human ovarian function: physiological concepts and clinical consequences. *Endocr. Rev*., 18: 71-106. https://doi.org/10.1210/edrv.18.1.0290

Griswold, M.D., Heckert, L. and Linder, C., 1995. The molecular biology of the FSH receptor. *J. Steroid Biochem. mol. Biol*., **53**: 215-218. https://doi.org/10.1016/0960-0760(95)00049-6

Gromoll, J., Pekel, E. and Nieschlag, E., 1996. The structure and organization of the human follicle-stimulating hormone receptor (*FSHR*) gene. *Genomics*, **35**: 308-311. https://doi.org/10.1006/geno.1996.0361

Heckert, L.L., Daley, I.J. and Griswold, M.D., 1992. Structural organization of the follicle-stimulating hormone receptor gene. *Mol. Endocrinol*., **6**: 70-80. https://doi.org/10.1210/mend.6.1.1738373

Wu, J.S., Zhang, G.X., Dai, G.J., Ye, L. and Wang, J.Y., 2012. Polymorphism of Exon10 of *FSHR* gene and its relationship with litter size in Xiaomeishan pigs. *Chin. J. Anim. Vet. Sci*., **43**: 509-515.

Kumar, A., 2017. *Molecular characterization of CYP19, FSHR and LHR genes and its association with reproduction traits in indigenous cattle*. PhD thesis, Rajasthan University, Bikaner, India.

Madrid, G.S., López, H.A. and Echeverri, Z.J.J., 2015. Association between FSHR polymorphism with productive and reproductive traits in Antioquia Holstein cattle. *Rev. Fac. Nac. Agron*., **69**: 7793-7801. https://doi.org/10.15446/rfna.v69n1.54747

Nagirnaja, L., Rull, K., Uusküla, L., Hallast, P., Grigorova, M. and Laan, M., 2010. Genomics and genetics of gonadotropin beta-subunit genes: Unique FSHB and duplicated LHB / CGB loci. *Mol. Cell. Endocrinol*., **329**: 4-16. https://doi.org/10.1016/j.mce.2010.04.024

Pan, X., Liu, S., Li, F., Wang, W., Li, C., Ma, Y. and Li, T., 2014. Molecular characterization, expression profiles of the ovine *FSHR* gene and its association with litter size. *Mol. Biol. Rep*., **41**: 7749-7754. https://doi.org/10.1007/s11033-014-3666-8

Patel, H., Bhartiya, D. and Parte, S., 2018. Further characterization of adult sheep ovarian stem cells and their involvement in neo-oogenesis and follicle assembly. *J. Ovarian Res*., **11**: 3. https://doi.org/10.1186/s13048-017-0377-5

Pérez-Solis, M.A., Macías, H., Acosta-Montesdeoca, A., Pasapera, A.M., Fierro, R., Ulloa-Aguirre, A. and Gutiérrez-Sagal, R., 2010. Molecular cloning and functional analysis of the FSH receptor gene promoter from the volcano mouse (*Neotomodon alstoni alstoni*). *Endocrine*, **37**: 98-105. https://doi.org/10.1007/s12020-009-9254-3

Rahal, P., Latronico, A.C., Kohek, M.B.F., Lucia, R.F.S.D., Milazzotto, M.P., Wheeler, M.B., Ferraz, J.B.S., Eler, J.P. and Garcia, A.J.F., 2010. Polymorphisms in the bovine follicle-stimulating hormone receptor gene. *Anim. Genet*., **31**: 280-281. https://doi.org/10.1111/j.1365-2052.2000.00604.pp.x

Richards, J.S., Jahnsen, T., Hedin, L., Lifka, J., Ratoosh, S., Durica, J.M. and Goldring, N.B., 1987. Ovarian follicular development: from physiology to molecular biology. *Recent Progr. Horm. Res*., **43**: 231-276. https://doi.org/10.1016/B978-0-12-571143-2.50012-5

Ruwanpura, S.M., Mclachlan, R.I. and Meachem, S.J., 2010. Hormonal regulation of male germ cell development. *J. Endocrinol*., **205**: 117-131. https://doi.org/10.1677/JOE-10-0025

Sacchi, S., Sena, P., Degli, E.C., Lui, J. and La, M.A., 2018. Evidence for expression and functionality of FSH and LH/hCG receptors in human endometrium. *J. Assist. Reprod. Genet*., **7**: 1-10.

Sairam, M.R. and Subbarayan, V.S., 2015. Characterization of the 5' flanking region and potential control elements of the ovine follitropin receptor gene. *Mol. Reprod. Dev*., **48**: 480-487. https://doi.org/10.1002/(SICI)1098-2795(199712)48:4<480::AID-

MRD8>3.3.CO;2-D

Sprengel, R., Braun, T., Nikolics, K., Segaloff, D.L. and Seeburg, P.H., 1990. The testicular receptor for follicle stimulating hormone: structure and functional expression of cloned cDNA. *Mol. Endocrinol.*, **4**: 525-530. https://doi.org/10.1210/mend-4-4-525

Sullivan, R.R., Faris, B.R., Eborn, D., Grieger, D.M., Cino-Ozuna, A.G. and Rozell, T.G., 2013. Follicular expression of follicle stimulating hormone receptor variants in the ewe. *Reprod. Biol. Endocrinol.*, **11**: 113-120. https://doi.org/10.1186/1477-7827-11-113

Ulloaaguirre, A., Zariñán, T., Pasapera, A.M., Casasgonzález, P. and Dias, J.A., 2007. Multiple facets of follicle-stimulating hormone receptor function. *Endocrine*, **32**: 251-263. https://doi.org/10.1007/s12020-008-9041-6

Wang, W., Liu, S., Li, F., Pan, X., Li, C., Zhang, X., Ma, Y., La, Y., Xi, R. and Li, T., 2015. Polymorphisms of the ovine BMPR-IB, BMP-15 and FSHR and their associations with litter size in two Chinese indigenous sheep breeds. *Int. J. mol. Sci.*, **16**: 11385-11397. https://doi.org/10.3390/ijms160511385

Wei, S., Chen, S., Gong, Z., Ouyang, X., An, L., Xie, K., Dong, J. and Min, W., 2013. Alarelin active immunization influences expression levels of GnRHR, FSHR and LHR proteins in the ovary and enhances follicular development in ewes. *Anim. Sci. J.*, **84**: 466-475. https://doi.org/10.1111/asj.12030

Wei, S., Shen, X., Gong, Z., Deng, Y., Lai, L. and Liang, H., 2017. FSHR and LHR expression and signaling as well as maturation and apoptosis of cumulus-oocyte complexes following treatment with FSH receptor binding inhibitor in sheep. *Cell Physiol. Biochem.*, **43**: 660-669. https://doi.org/10.1159/000480650

Xu, Z., Garverick, H.A., Smith, G.W., Smith, M.F., Hamilton, S.A. and Youngquist, R.S., 1995. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol. Reprod.*, **53**: 951-957. https://doi.org/10.1095/biolreprod53.4.951

Zilaitiene, B., Dirzauskas, M., Verkauskiene, R., Ostrauskas, R., Gromoll, J. and Nieschlag, E., 2018. The impact of FSH receptor polymorphism on time-to-pregnancy: a cross-sectional single-centre study. *BMC Pregn. Childb.*, **18**: 272-279. https://doi.org/10.1186/s12884-018-1910-2