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Polymorphism of 5′ regulatory region of ovine FSHR gene and its association with litter size in Small Tail Han sheep

M. X. Chu · X. H. Guo · C. J. Feng · Y. Li · D. W. Huang · T. Feng · G. L. Cao · L. Fang · R. Di · Q. Q. Tang · Y. H. Ma · K. Li

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Abstract Single nucleotide polymorphisms of 5′ regulatory region of follicle-stimulating hormone receptor (*FSHR*) gene were detected in two high prolificacy sheep breeds (Small Tail Han and Hu sheep) and two low prolificacy sheep breeds (Corriedale and Chinese Merino sheep) by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). The results indicated that there were three genotypes (AA, AB and BB) detected by primer 1 in Hu sheep while only one genotype (AA) in other three sheep breeds, and frequencies of AA, AB and BB genotypes in Hu sheep were 0.700, 0.225 and 0.075, respectively. There were three genotypes (EE, EF and EG) detected by primer 3 in Small Tail Han sheep while only EE genotype occurred in other three sheep breeds, and frequencies of EE, EF and EG genotypes in Small Tail Han sheep were 0.775, 0.200 and 0.025, respectively. No polymorphism was detected in four sheep breeds by primer 2 and primer 4. The sequencing results showed that there were two nucleotide mutations (g. −681T>C and g. −629C>T) in genotype BB compared with AA for primer 1. As for primer 3, two mutations (g. −197G>A and g. −98T>C) in genotype EF compared with EE and two mutations (g. −200G>A and g. −197G>A) in genotype EG compared with EE. The heterozygous ewes with EG or EF had 0.89 ($P < 0.05$) or 0.42 ($P < 0.05$) lambs more than homozygous ewes (EE genotype) in Small Tail Han sheep, respectively, while there was no significant difference on litter size between EG and EF ewes.

Keywords Sheep · Litter size · Follicle-stimulating hormone receptor gene · PCR-SSCP

Introduction

Follicle stimulating hormone (FSH) is secreted by anterior pituitary and plays a key role in reproduction [1], which was proved to be critical for follicle growth, development, differentiation and triggering the maturation and ovulation of follicles. As FSH is a kind of biological macromolecules which can not permeate through the cell membrane, so its effect on target cells must be mediated by FSH receptor (FSHR), a member of the family of G-protein-coupled receptors expressed solely in granulosa cells [2–4]. So far, *FSHR* cDNA of many species have been cloned and sequenced such as human, cattle, sheep, pig and so on, but the 5′ flanking region sequence of sheep *FSHR* gene is still unknown. *FSHR* cDNA was first cloned from rat Sertoli cell in 1990 and its genetic mutations may affect the ability of FSH signal transduction [5]. Abdennebi et al. [6] found that both *FSHR* mRNA levels and responsiveness of viable

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M. X. Chu (✉) · D. W. Huang · T. Feng · G. L. Cao · L. Fang · R. Di · Q. Q. Tang · Y. H. Ma · K. Li
Key Laboratory of Farm Animal Genetic Resources and Utilization of Ministry of Agriculture, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China
e-mail: mxchu@263.net

X. H. Guo
Department of Physiology and Pathophysiology, Peking University Health Science Center and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing 100191, People's Republic of China

C. J. Feng · Y. Li
College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471003, People's Republic of China

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*. In addition, variants in *FSHR* gene were proved to be associated with reproductive diseases in human such as polycystic ovary syndrome (PCOS) [7], primary and secondary amenorrhea [8] and male infertility [9].

There were four breeds of sheep in the present study. Mean live litter sizes of Small Tail Han sheep of Shandong province, Hu, Chinese Merino and Corriedale sheep have been reported to be 2.61, 2.29, 1.17–1.28, 1.21–1.30, respectively [10]. The objectives of the present study were firstly to detect single nucleotide polymorphisms (SNPs) in 5′ flanking region of *FSHR* gene in both two high prolificacy breeds (Small Tail Han and Hu sheep) and two low prolificacy breeds (Chinese Merino and Corriedale sheep) by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP), and secondly to investigate the association between *FSHR* gene and prolificacy in sheep breeds in which the polymorphism is segregating so as to acquire molecular markers related with prolificacy for marker assisted selection.

Materials and methods

Animals and reagents

Venous jugular blood samples (10 ml per ewe) were collected from 252 Small Tail Han ewes lambed in 2008, along with data on litter size in the first three parities (Jiaxiang Sheep Breeding Farm located in Jiaxiang County, Shandong Province, P. R. China), 56 Corriedale ewes (Qinshui Demonstration Farm located in Qinshui County, Shanxi province, P. R. China), 72 Chinese Merino ewes (Ziniquan Breeding Sheep Farm located in Shihezi City, Xinjiang Uygur Autonomous Region, P. R. China) and 80 Hu ewes (Yuhang Hu Sheep Breeding Farm located in Yuhang District, Hangzhou City, Zhejiang province, P. R. China) using acid citrate dextrose (ACD) as an anticoagulant. Genomic DNA was extracted from whole blood by phenol–chloroform method, and then dissolved in TE buffer [10 mmol/l Tris–HCl (pH 8.0), 1 mmol/l EDTA (pH 8.0)] and kept at −20°C. All experimental procedures were performed according to authorization granted by the Chinese Ministry of Agriculture.

The 252 Small Tail Han sheep ewes were selected at random and they were the progeny of five rams (n = 47, 49, 51, 52, 53). Because the five rams had been sold, their blood was not collected for genotyping. No selection on litter size or other fertility traits was performed in the flock over previous years. Lambing seasons consisted of 3-month groups starting with March through to May as season 1 (spring, n = 65), June through to August as 2 (summer, n = 59), September through to November as 3 (autumn, n = 72) and December through to February as 4 (winter, n = 56).

Main reagents (such as *Taq* DNA polymerase, dNTPs, pGM-T vector, *Escherichia coli* DH5α and PCR purification kits, etc.) were purchased from Tiangen (Beijing) Ltd. Co., Beijing, P. R. China.

Primers and PCR amplification

According to the promoter region, exon 1 and partial cds sequence of *Ovis aries FSHR* gene (GenBank No. AF090438), four pairs of primers (listed in Table S1 [see Supplemental data]) were used to amplify partial of the 5′ flanking region of the ovine *FSHR* gene. The primers were synthesized by Shanghai Invitrogen Biotechnology Ltd Co. (Shanghai, P. R. China). PCRs were carried out in 25 μl of reaction mixture containing 2.5 μl of 10× PCR buffer, 2.0 μl of 2.5 mmol/l dNTP mixture, 0.2 μl of 10 μmol/l of each primer, and 1 U Taq polymerase, 3.0 μl of 50 ng/μl genomic DNA and 1.5 μl of 25 mmol/l Mg^{2+}, the rest was ddH_2O. PCR conditions for all four pairs of primers were as follows: initial denaturation at 95°C for 7 min, followed by 31 cycles of denaturation at 94°C for 30 s, annealing for 30 s (annealing temperatures were listed in Table S1 [see supplemental data]), extension at 72°C for 30 s, with a final extension at 72°C for 10 min. Then, the products of amplification were identified on 1.5% agarose gels and kept at 4°C.

SSCP detection

SSCP analysis of amplified fragments was used to detect polymorphisms. A volume of 2.0 μl PCR product was mixed with 7.0 μl gel loading solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 20 mmol/l EDTA (pH 8.0) and 10% glycerol. The mixture was denatured at 98°C for 10 min, then cooled on ice for 7 min and loaded on 12–14% (14% for primers 1 and 3, 12% for primers 2 and 4) neutral polyacrylamide gels (acrylamide:bisacrylamide = 39:1 for primers 2, 3 and 4; 49:1 for primer 1). Electrophoresis was performed at 140 V at 4°C overnight (run times for primers 1 to 4 were 16, 15.5, 15, 15 h, respectively). After electrophoresis, the DNA fragments in the gels were visualized by silver staining, photographed and analyzed by an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Cloning and sequencing

After SSCP analysis, the PCR products of different genotypes were separated on 1% agarose gels and recovered using PCR purification kits. Each DNA fragment was ligated into the pGM-T vector, and then was transformed into *E. coli* DH5α competent cells. Positive clones of transformed cells were identified by restriction enzyme digestion. The target DNA fragments in recombinant plasmids were sequenced from both directions using an ABI3730 automatic sequencer by Shanghai Invitrogen Biotechnology Ltd. Co. (Shanghai, P. R. China).

Statistical analysis

The following fixed effects model was employed for analysis of litter size in Small Tail Han ewes and least squares mean was used for multiple comparison in litter size among different genotypes.

$$y_{ijklm} = \mu + S_i + LS_j + P_k + G_l + e_{ijklm},$$

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; 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 8.1) (SAS Institute Inc., Cary, NC, USA). Mean separation procedures were performed using a least significant difference test.

Results

PCR-SSCP analysis

Genomic DNA of four sheep breeds was amplified using four pairs of primers for *FSHR* gene, in which the fragments amplified by primers 1 and 3 were genetically polymorphic, whereas others showed no polymorphism. Three genotypes for both primer 1 and primer 3 were identified by PCR-SSCP, for primer 1 they were defined as AA, AB and BB (Fig. 1), and for primer 3 were EE, EF and EG, respectively (Fig. 2).

Sequencing of different genotypes

To determine the mutations, two homozygous genotypes for primer 1 and three genotypes for primer 3 were cloned and sequenced. The sequencing results were analyzed by DNAMAN, Chromas and BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Sequence analysis revealed that there were two nucleotide mutations (g. −681T>C and g. −629C>T) in genotype BB compared with AA for primer 1 (Fig. 3). As for primer 3, two mutations (g. −197G>A and g. −98T>C) in genotype EF compared with EE and two mutations (g. −200G>A and g. −197G>A) in genotype EG compared with EE (Fig. 4).

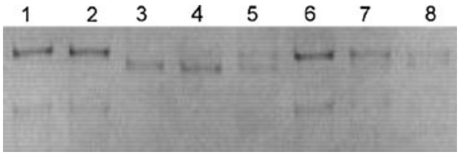

Fig. 1 SSCP analysis of PCR amplification using primer 1 in Hu sheep (14% polyacrylamide gel stained with silver nitrate). *Lanes 1*, *2*, *6*: AA genotype; *Lanes 3*, *4*: BB genotype; *Lanes 5*, *7*, *8*: AB genotype

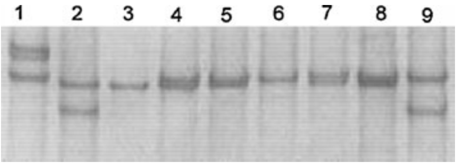

Fig. 2 SSCP analysis of PCR amplification using primer 3 in Small Tail Han sheep (14% polyacrylamide gel stained with silver nitrate). *Lane 1*: EG genotype; *Lanes 2*, *9*: EF genotype; *Lanes 3–8*: EE genotype

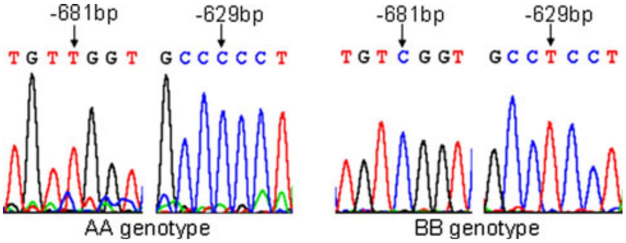

Fig. 3 Sequence comparison of AA and BB genotypes at the 114th and 166th bp of cDNA of *FSHR* gene using primer 1 in Hu sheep

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

As listed in Table 1, for primer 1, polymorphisms were found only in prolific Hu sheep and frequencies of AA, AB and BB genotypes were 0.700, 0.225 and 0.075, respectively, whereas only one genotype (AA) in prolific Small Tail Han, nonprolific Corriedale and Chinese Merino sheep. For primer 3, three genotypes EE, EF and EG only existed in prolific Small Tail Han and the frequencies were 0.775, 0.200 and 0.025, respectively, while there was only one genotype EE in other three sheep breeds.

Influence of fixed effects on litter size in Small Tail Han sheep

The least squares mean and standard error for litter size of different *FSHR* genotypes in Small Tail Han sheep were given in Table 2. The heterozygous ewes with EG or EF

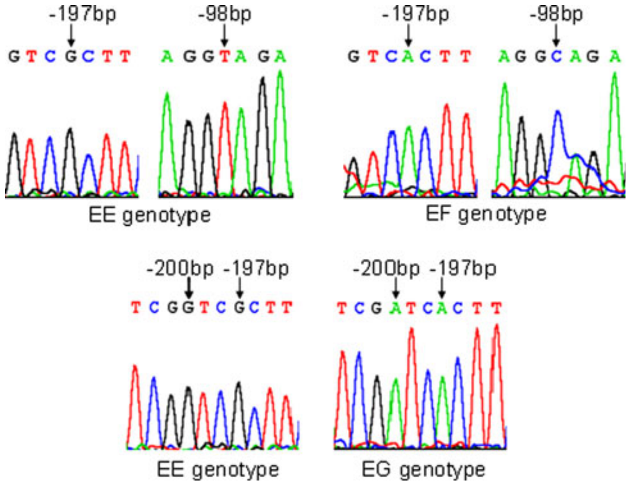

Fig. 4 Sequence comparison of EE, EF and EG genotypes of cDNA of *FSHR* gene using primer 3 in Small Tail Han sheep

Table 1 Allele and genotype frequencies of *FSHR* gene in four sheep breeds

Breed	Small Tail Han sheep	Hu sheep	Chinese Merino sheep	Corriedale sheep
Primer 1				
Number	252	80	72	56
Genotype frequency				
AA	1.000(252)	0.700(56)	1.000(72)	1.000(56)
AB	0.000(0)	0.225(18)	0.000(0)	0.000(0)
BB	0.000(0)	0.075(6)	0.000(0)	0.000(0)
Allele frequency				
A	1.000	0.812	1.000	1.000
B	0.000	0.188	0.000	0.000
Primer 3				
Number	240	80	72	56
Genotype frequency				
EE	0.775(186)	1.000(80)	1.000(72)	1.000(56)
EF	0.200(48)	0.000(0)	0.000(0)	0.000(0)
EG	0.025(6)	0.000(0)	0.000(0)	0.000(0)
Allele frequency				
E	0.888	1.000	1.000	1.000
F	0.100	0.000	0.000	0.000
G	0.012	0.000	0.000	0.000

Note The numbers in the parentheses are the individuals that belong to the genotypes, respectively

genotype had 0.89 ($P < 0.05$) or 0.42 ($P < 0.05$) lambs more than the homozygous EE ewes, while there was no significant difference on litter size between EG and EF ewes.

Table 2 Least squares mean and standard error for litter size of different *FSHR* genotypes in Small Tail Han sheep

Genotype	Number of samples	Litter size
EE	186	$2.06^{a} \pm 0.08$
EF	48	$2.48^{b} \pm 0.12$
EG	6	$2.95^{b} \pm 0.14$

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

Discussion

Polymorphisms of 5′ flanking region of *FSHR* gene

So far, there have been a large number of studies of *FSHR* gene as a candidate gene on reproductive traits in animals, which revealed that *FSHR* gene plays an important role in animal reproduction. A SNP (−739G → T) in 5′ flanking region of Boer goat *FSHR* gene was initially detected by PCR-SSCP [11]. And five mutations (−93C → A, −80G → C, −63C → A, −56C → G and −55T → C) were identified in Xiangdong Black, Nanjiang Brown and Guizhou Black goats [12]. No mutations was detected at several potential loci of transcriptional regulating elements (such as CRE, ERE and Y-box type) in 5′ flanking region of *FSHR* gene in Australian sheep [13], Small Tail Han sheep [14] and Chinese Simmental Cattle [15]. Recently, several polymorphisms were identified in 5′ flanking region of *FSHR* gene. A common mutation (−29G → A) in 5′ flanking region of *FSHR* gene was detected in both women with primary and secondary amenorrhea in India [8] and infertile men in Southeast Turkey [9]. Besides, mutation −278G → A was found in Chinese Holstein cows [16].

In the present study, two novel SNPs (g. −681T>C and g. −629C>T) were detected in 5′ flanking region of *FSHR* gene in Hu sheep, and other three novel SNPs (g. −200G>A, g. −197G>A and g. −98T>C) were found in 5′ flanking region of *FSHR* gene in Small Tail Han sheep.

Effect of *FSHR* gene on reproductive performance

Many studies showed that *FSHR* gene had a great impact on reproductive performance of human and animals. There was a C566T transition in exon 7 of human *FSHR* predicting an Ala to Val substitution at residue 189 in the extracellular ligand-binding domain. Though the ligand-binding affinity of the mutated receptor was apparently normal, expression of the gene in transfected cells demonstrated a dramatic reduction of binding capacity and signal transduction. C566T mutation may cause hereditary hypergonadotropic ovarian failure or ovarian dysgenesis (ODG) in multiplex affected families of Finland [17]. The same mutation also existed in swine *FSHR* and the polymorphism was significantly associated with total born number and number born alive in Erhualian and Yorkshire sows [18]. Additionally, the mutation rate of exon 10 of *FSHR* gene between monovular and twinning cows was significantly different, *FSHR* gene may be a candidate gene of fecundity in Qinchuan and Holstein cattle breeds [19].

Several mutations in 5′ regulatory region of *FSHR* gene also had an effect on reproduction in mammals. Correlation analysis between 5′ regulatory region of goat *FSHR* gene and the litter size revealed that *FSHR* gene had no significant effect on the litter size in both Xiangdong Black goats and Nanjiang Brown goats ($P > 0.05$), but had significant effect on the litter size in Guizhou Black goats ($P < 0.05$), and the litter size of does with BB genotype was significantly higher than that of AA and AB genotype [12]. The AA genotype of −29G → A mutation was proved to be associated with increased serum FSH levels in the Indian women with primary or secondary amenorrhea [8], but this mutation did not influence the serum FSH levels of infertile men in Southeast Turkey [9]. As for polymorphic locus −278 in *FSHR* gene of Chinese Holstein cows, all cows absence of superovulation response were genotyped as CD and DD, while cows with genotype CC had a significant increase in the total number of ova (TNO) ($P < 0.01$), and produced more number of transferable embryos (NTE) than those with genotypes CD and DD ($P < 0.01$) [16].

In conclusion, we found four mutations in the 5′ regulatory region of sheep *FSHR* gene. The distribution of SNPs of the *FSHR* gene in sheep breeds is evidently different and the *FSHR* gene may affect the litter size of sheep significantly. Since breeds and population sizes of sheep were relatively small, the results of the present study were preliminary. Although the present study had firstly demonstrated the effect of 5′ regulatory region of sheep *FSHR* gene on the litter size, further studies of association between *FSHR* gene and reproductive performance are required with increasing breeds and individuals of sheep.

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