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Novel single nucleotide polymorphisms of *GnRHR* gene and their association with litter size in goats

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Abstract

In the present study, the polymorphisms of gonadotropin-releasing hormone receptor (*GnRHR*) gene were analysed as a genetic marker candidate for litter size in 720 individuals from Shaanan goats (SG) and Boer goats (BG). Two alleles (A and C), two observed genotypes (AA and AC), and single nucleotide polymorphisms (SNPs) were detected. The frequencies of alleles A and C in two goat breeds were 0.78-0.82 and 0.18-0.22, respectively. The SNP locus was in Hardy–Weinberg disequilibrium in two goat breeds ($P<0.05$). In addition, comparisons between the nucleotide sequences of AA and AC genotypes showed one mutation (T>A) at exon 2. The results showed that AA genotype was associated with better litter size in SG and BG breeds. Therefore, these results suggest that *GnRHR* gene is a strong candidate gene that affects litter size in goats.

Keywords: *GnRHR*, polymorphisms, litter size, goat

Zusammenfassung

Neue Einzelnukleotid-Polymorphismen des *GnRHR*-Gens und deren Assoziation mit der Wurfgröße bei Ziegen

In der vorliegenden Studie wurden die Polymorphismen des Gonadotropin-releasing hormone receptor (*GnRHR*) Gens in 720 Ziegen der Rassen Shaanan und Boer als Genmarker Kandidat für die Wurfgröße untersucht. Zwei Allele (A und C), zwei beobachtete Genotypen (AA und AC) und Einzelnukleotid-Polymorphismen wurden entdeckt. Die Frequenzen der Allele A und C in den beiden Ziegenrassen waren 0,78-0,82 und 0,18-0,22. Der SNP-Locus befand sich im Hardy-Weinberg-Ungleichgewicht in beiden Rassen ($P<0.05$). Vergleiche zwischen den Nukleotidsequenzen von AA und AC Genotypen zeigten eine Mutation (T>A) bei Exon 2. Im Ergebnis kann der AA Genotyp mit einer besseren Wurfgöße in Verbindung gebracht werden. Das *GnRHR*-Gen könnte daher ein starkes Kandidatengen sein, dass die Wurfgröße von Ziegen beeinflusst.

Schlüsselwörter: *GnRHR*, Polymorphismen, Wurfgröße, Ziege

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Introduction

In animal industry, reproductive traits of animal are always of primary concern during breeding for its determinant economical value. However, improvement of reproductive traits in goat by traditional selective breeding has proved to be difficult due to the low heritability for litter size (An *et al.* 2010). The candidate gene approach, employed in identifying the polymorphisms in genes likely to cause phenotypic variation based on physiological and biochemical evidence, could accelerate the improvement of goat reproductive traits. With the development of candidate genes and comparative mapping approaches, some major genes affecting important economic traits in sheep have been successfully identified such as the bone morphogenetic protein receptor-IB gene which is described as the successful proof of an association between a candidate gene and litter size (Wilson *et al.* 2001, Mulsant *et al.* 2001).

Gonadotropin-releasing hormone receptor (*GnRHR*) is a member of the rhodopsin-like G protein-coupled receptor (GPCR) family and predominantly couples the Gq/11 family of G proteins in various cellular environments (Stojilkovic *et al.* 1994). GPCRs are characterized structurally as seven transmembrane-spanning helices, linked by consecutive extracellular and intracellular loops, and bearing an extracellular amino-terminal domain, which may be glycosylated, and a cytoplasmic carboxyl-terminal tail, which may be palmitoylated. In general, the extracellular domains and/or transmembrane regions are involved in the formation of the ligand-binding pocket, whereas the cytoplasmic regions present sites for interactions with G proteins and other intracellular regulatory proteins (Stojilkovic *et al.* 1994, Sealfon *et al.* 1997). *GnRHR* binds with high affinity to GnRH on pituitary gonadotropes to stimulate release of the gonadotropic hormones; luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that in turn regulate production of gametes and gonadal hormones (Naor 2009). The interaction of GnRH and its receptor is a critical event in the endocrine control of reproduction. The *GnRHR* is expressed in the pituitary, the gonads, and the hypothalamus (Huang *et al.* 2001, Ikemoto & Park 2005, Rhee *et al.* 2008). Since the *GnRHR* occurs in the gonads as well as in the pituitary, its effect might occur at the level of the ovary (Kang *et al.* 2001), possibly by affecting cell proliferation and apoptosis as suggested in mammals (Takekida *et al.* 2000). Until now, the association of *GnRHR* genetic variations with litter size has not been reported in goat. The *GnRHR* gene is associated with secretion of luteinizing hormone and follicle-stimulating hormone; therefore, it may be a potential candidate gene for litter size in goat. The objectives of this study were to search for SNPs of *GnRHR* gene and evaluate the associations between *GnRHR* polymorphisms and litter size.

Materials and methods

DNA samples

All procedures involving animals were approved by Boer goat breeding centers and Shaanan Green Century Biology Development Company in Shaanxi Province, China. A total of 720 female goats were examined, including 430 Shaanan goats (SG) and 290 Boer goats (BG). Records of litter size for different goat breeds were collected for statistical analysis. Approximate 5 ml blood per goat was collected aseptically from the jugular vein and kept

in a tube containing anticoagulant ACD (10:27:38 Citric acid:Sodium citrate:C6H12O6). All samples were delivered back to the laboratory in an ice box. The genomic DNA was extracted from white blood cells using standard phenol-chloroform extraction protocol (Sambrook & Russell 2001, Muszyńska *et al.* 2010). The DNA samples were dissolved in TE buffer which was made from 10 mM Tris–Cl (pH7.5) and 1 mM EDTA (pH 8.0) and were stored at –20 °C for use.

PCR conditions

According to the sequence of sheep *GnRHR* gene (GenBank acc. no. L42937 and L43841), a pair of primers (F: 5-CCT ACA GTT ATA CAT CTT TGG GA-3, R: 5-GAG AAA TAC ATA CTG TGG GGA T-3) were designed to amplify 241 bp exon 2 of *GnRHR* gene. The 25 µL volume contained 50 ng genomic DNA, 0.5 µM of each primer, 10× buffer (including 1.5 mM $MgCl_2$), 200 µM dNTPs and 0.5 units of Taq DNA polymerase (MBI). The cycling protocol was 4 min at 95 °C, 35 cycles of denaturing at 94 °C for 30 s, annealing at 55.8 °C for 30 s, extending at 72 °C for 30 s, with a final extension at 72 °C for 10 min.

Single strand conformation polymorphism (SSCP) and DNA sequencing

PCR products (5 µl) were mixed with 5 µl denaturing solution (95 % formamide, 25 mM EDTA, 0.025 % xylene-cyanole and 0.025 % bromophenol blue), heated for 10 min at 98 °C and chilled on ice. Denatured DNA samples were subjected to PAGE (80×73×0.75 mm) in 1× TBE buffer and constant voltage of 200 V for 3.5 h. The gel (29:1 acrylamide:bis) was stained with 0.1 % silver nitrate (An *et al.* 2009). After the polymorphisms were detected, the PCR products of different electrophoresis patterns were sent to sequence in both directions in ABI 377 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and the sequences were analysed with DNAstar Lasergene 7.1 (Madison, WI, USA) and BBLAST (National Center for Biotechnology Information, Bethesda, MD, USA).

Statistical analysis

The genotypic frequencies, heterozygosity (He) and polymorphism information content (PIC) were calculated using cluster-analysis software v 1.2 (Poultry Research Institute, Yangzhou, China). Associations of the genotypes with litter size of goats were determined by the analysis of variance of quantitative traits using SPSS 16 (SPSS Inc., Chicago, IL, USA). The association analysis was done separate for each breed. Each trait was analysed using the multiple trait derivative-free restricted maximum likelihood (MTDFREML) computer programs (Boldman *et al.* 1995). Pedigrees of goats were traced back three generations to create the numerator relationship matrix. All analyses were done in two steps, first using a full animal model and then using a reduced animal model. The full animal model included fixed effects of marker genotype, parity, season of birth (spring vs. fall), sire, farm and random effects of animal (Ge *et al.* 2003). The reduced linear model was used in the final analysis and included with fixed effects was established and included effects of sire, dam within sire, as well as interaction between parity and genotype. The reduced model applied was:

$$Y_{hlikm} = \mu + S_h + D_{lh} + G_i + P_k + (PG)_{ki} + E_{hlikm} \quad (1)$$

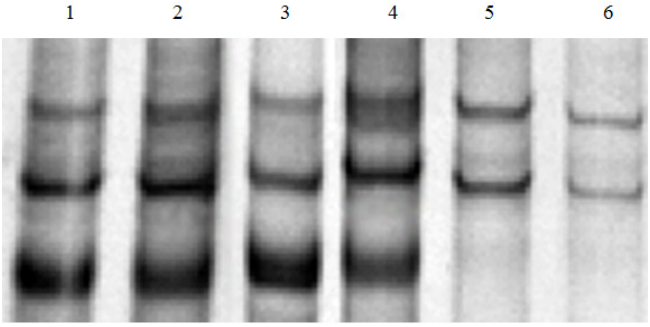
where Y_{hlikm} is the trait measured on each of the *hlikm*-th animal, μ is the overall population

mean, S_h was the fixed effect associated with the *h*-th sire, D_{lh} was the fixed effect associated with *l*-th dam with sire *h*, G_i is the fixed effect associated with ith genotype, P_k is the fixed effect associated with the *k*-th parity, $(PG)_{ki}$ is interaction between the *k*-th parity and the ith genotype, and Ehlikm is the random error. An effect associated with farm and season of birth (spring versus fall) is not matched in the linear model, as the preliminary statistical analyses indicated that these effects did not have a significant influence on variability of traits in analysed populations.

Results

Polymorphisms of GnRHR gene in two goat breeds

According to international practice about the naming of SSCP patterns (Cerit *et al.* 2004, Gupta *et al.* 2009, Kulig *et al.* 2009), different SSCP patterns were named AA and AC genotypes, respectively (Figure 1). CC genotype was not detected because of a lower frequency. The alleles were named A and C, respectively. AA and AC genotypes were found in two goat breeds. Frequencies of A allele were 0.78 and 0.82, and frequencies of C allele were 0.18 and 0.22, and the PIC was 0.25 and 0.28, respectively in SG and BG breeds. Genotypic frequencies, He and the equilibrium χ2-test are shown in Table 1. The SNP locus was in Hardy-Weinberg disequilibrium in both breeds, respectively ($P<0.05$). The different SSCP patterns AA and AC of *GnRHR* gene exon 2 amplified by the primer shown in Figure 1 were sequenced in both directions. Comparisons between both nucleotide sequences show one mutation (T>A) at exon 2.



1, 2, 3, 4: AC genotype,
5, 6: AA genotype

Figure 1
SSCP analysis of PCR products using primer in two goat breeds

Table 1
Genetic structure of *GnRHR* gene for different breeds

Breeds	Observed genotypes		Genotypic frequencies		Allelic frequencies		He	PIC	Equilibrium χ^2-test
	AA	AC	AA	AC	A	C			
SG	240	190	0.56	0.44	0.78	0.22	0.34	0.28	5.81 ($P<0.05$)
BG	187	103	0.64	0.36	0.82	0.18	0.30	0.25	24.33 ($P<0.05$)

Association of polymorphisms with litter size in two goat breeds

Litter size was analysed in SG and BG breeds. Sample size of AA genotype was 240 and 187 goats, and that of AC genotype was 190 and 103 goats, respectively in SG and BG breeds. In SG breeds, the goats with AA genotype had greater litter size than those of AC genotype

($P<0.01$) from the first to third parity. In BG breeds, the goats with AA genotype had greater litter size than those of AC genotype ($P<0.01$) at the second, third and fourth parity. In addition, the goats with AA genotype had greater average litter size than those of AC genotype (Table 2). The rest of the records of litter size had no significant association.

Table 2
Association of *GnRHR* genotypes with litter size (mean±SE) in SG and BG breeds

Breeds	Genotypes	Sample size	1st parity litter size	2nd parity litter size	3rd parity litter size	4thparity litter size	Average litter size
SG	AA	240	1.67 ± 0.03^{A}	2.02 ± 0.03^{A}	2.12 ± 0.02^{A}	2.26 ± 0.03^{A}	2.02 ± 0.01^{A}
	AC	190	1.48 ± 0.04^{B}	1.79 ± 0.03^{B}	1.88 ± 0.04^{B}	2.12 ± 0.04^{A}	1.82 ± 0.02^{B}
BG	AA	187	1.29 ± 0.03^{A}	1.80 ± 0.04^{A}	1.86 ± 0.04^{A}	2.01 ± 0.04^{A}	1.74 ± 0.02^{A}
	AC	103	1.37 ± 0.05^{A}	1.54 ± 0.05^{B}	1.66 ± 0.05^{B}	1.64 ± 0.05^{B}	1.55 ± 0.03^{B}

^{A,B,C}different superscripts differ significantly at $P<0.01$

Discussion

Gonadotropin-releasing hormone plays a critical role in the control of reproductive functions in mammals by stimulating the biosynthesis and secretion of the gonadoropins (luteinizing hormone, LH and follicle-stimulating hormone, FSH) from the pituitary (Kumar & Trant 2001, Kah *et al.* 2007). In the human genome, two forms of GnRH have been identified, GnRH-I (mammal GnRH) and GnRH-II (chicken GnRH II). Both forms and their common receptor are expressed, apart from the hypothalamus, in various compartments of the human ovary. Gonadal steroids, gonadotropins, and GnRH itself controls the regulation of the *GnRH*/*GnRHR* system gene expression in the human ovary (Cheng *et al.* 2001, Yeung *et al.* 2005, Metallinou *et al.* 2007). In addition, the *GnRHR* protein is known to be involved in different developmental and metabolic processes and different expression in some of them are responsible for ovarian diseases (Choi *et al.* 2006, Wilkinson *et al.* 2008). However, how they influence diseases is not exactly known. It is possible that the mutations influence sex hormone levels and this, in turn, leads to disease. Sun *et al.* (2008) reported two mutations (A50G and A101C) of *GnRHR* exon 2 in DD genotype compared with CC genotype in Small Tail Han Sheep, and the goats with DD genotype had more litter size than those of CC genotype. Jiang *et al.* (2001) reported a F2 population of Meishan × European Large White pigs was genotyped for a TG deletion/insertion in the promoter region of *GnRHR* gene, and a C/G substitution in the 3UTR (untranslated region). A significant association of the C/G substitution with the number of corpora lutea at first parity was observed. Dunn *et al.* (2004) found an additive effect of *GnRHR* gene on the number of double-yolked eggs ($P<0.05$) in one generation of a commercial broiler breeder hen population. Genetic variants within the *GnRHR* gene have been previously investigated as candidates for egg-laying traits in Chicken (Wu *et al.* 2007). Their results showed that *GnRHR* gene has a significant effect on reproduction. This study is a preliminary report on a novel SNP of *GnRHR* gene in 720 goats by PCR-SSCP and DNA sequencing methods. The present study revealed that polymorphisms of the *GnRHR* gene are significantly associated with litter size in SG and BG breeds, and showed that genotype AA might be associated with better litter size in both breeds. However, further analysis should be performed in order to validate both the association and the physiological significance of the mutation in the exon 2 of *GnRHR* gene.

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