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Polymorphism in exon 2 of *INHBB* gene and its relationship with litter size in Jining Grey goats*

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The inhibin β_B *(INHBB)* gene was studied as a candidate gene for the prolificacy of Jining Grey goats. According to the sequence of bovine *INHBB* gene, two pairs of primers were designed to detect single nucleotide polymorphism in exon 2 of *INHBB* gene in high-prolificacy (Jining Grey) and low-prolificacy (Inner Mongolia Cashmere and Angora) goats by polymerase chain reaction-single strand conformation polymorphism method. Only the amplified products of primer P2 showed polymorphism. Three genotypes (AA, AB and BB) were identified in Jining Grey and two (AB and BB) in Inner Mongolia Cashmere and Angora goats. Sequencing revealed one single nucleotide mutation (A→G) at base 782 of exon 2 of *INHBB* gene in BB genotype compared to genotype AA.

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In Jining Grey goats the frequencies of A and B alleles were 0.318 and 0.682 while of AA, AB and BB genotypes reached 0.068, 0.500 and 0.432, respectively. The Jining Grey goats remained in Hardy-Weinberg equilibrium (chi-square = 3.36, P = 0.1861). These of genotype AA delivered by 0.73 (P<0.05) or 0.84 (P<0.05) kids more than those with genotype AB or BB, while does of genotype AB – by 0.11 (P>0.05) kids more than did does of genotype BB.

KEY WORDS: gene polymorphism / goat / Inhibin β_B gene / PCR-SSCP / prolificacy

Inhibin is a glycoprotein hormone belonging to the transforming growth factor-β superfamily that suppresses follicle-stimulating hormone (FSH) synthesis and secretion [Ling *et al*. 1985, Rivier *et al*. 1985, Robertson *et al*. 1985, Woodruff *et al*. 1996]. It consists of two subunits, α and β, linked by disulphide bonds. Two inhibins, sharing a common α-subunit but different subunits-β (β_A or β_B) had been identified [Mason *et al*. 1985]. The hormone is expressed in ovary, testis and uterus of human, mouse, pig, and sheep [Phillips 2005]. In sheep, a major source of inhibin are follicles [Rodgers *et al*. 1989] but there is evidence for its extraovarian sources as well [McNatty *et al*. 1992]. In goats inhibin β_B gene *(INHBB)* had been mapped to chromosome 2q31→q33 [Goldammer *et al*. 1995]. Female mice carrying mutations in the *INHBB* gene suffered from distinct developmental and reproductive defects [Vassalli *et al*. 1994]. *INHBB* had significant effect on litter size in some sheep breeds [Jaeger and Hiendleder 1994]. Polymorphism of goat *INHBB* has not been reported so far.

The Jining Grey goat that has significant characteristics of sexual precocity, year-round oestrus, and high prolificacy is an excellent local breed of Popular Republic of China [Tu 1989]. The mean live litter sizes of Jining Grey, Inner Mongolia Cashmere and Angora goats have been reported to be 2.94 [Tu 1989], 1.04 [Tu 1989] and 1.31 [Roberts and Reeves 1988], respectively. Based on the important role of inhibin in reproduction, its gene was considered as a possible candidate gene for the prolificacy of Jining Grey goats. The objectives of the present study were firstly to detect single nucleotide polymorphism (SNP) in exon 2 of *INHBB* in both high (Jining Grey) and low (Inner Mongolia Cashmere and Angora) prolificacy goat breed by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) method, and secondly to investigate the association between SNP of *INHBB* gene and prolificacy in Jining Grey goats in which the polymorphism is segregating.

Material and methods

All procedures involving animals were approved by the Local Committees for Ethics in Experimentation on Animals and were approved and authorized by the Chinese Ministry of Agriculture.

Venous jugular blood samples (10 ml per goat doe) were collected from 146 Jining Grey goat does kidded in 2007, along with data on their litter size in the first, second and third parity (Jining Grey Goats Conservation Base, Jiaxiang County, Shandong Province, China), 38 Angora goat does (Qinshui Demonstration Farm, Qinshui County,

Shanxi Province, China), and 40 Inner Mongolia Cashmere goat does (Inner Mongolia White Cashmere Goat Breeding Farm, Etuokeqi, Ordos City, the Inner Mongolia Autonomic Region, China) using acid citrate dextrose as an anticoagulant. Genomic DNA was extracted from whole blood by phenol-chloroform method as described by Sambrook and Russell [2001], 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.

The 146 Jining Grey goats were confirmed to be progeny of five sires with detailed pedigree data. However when we selected the 146 goats for this study, the five sires had been killed and sold and we can't get their blood.)

No selection on litter size or other fertility traits was performed in the flock over previous years. Kidding seasons consisted of four 3-month periods: March, April, May as season 1 (spring), June, July, August as season 2 (summer), September, October, November as season 3 (autumn) and December, January, February as season 4 (winter).

Two pairs of primers were designed according to the sequence of exon 2 (GenBank accession number U16241) of bovine *INHBB* gene published by Thompson *et al*. [1994]. The primers were synthesized by SHANGHAI INVITROGEN BIOTECHNOLOGY, Ltd., Shanghai, China. The expected amplification fragment size was 244 bp for primer P1 and 296 bp for primer P2. The primer sequences used were as follows: Primer P1: F: 5'- CGGGTCCGCCTGTACTTCTT-3'; R: 5'-CCTGGATGGGTTCGGTGAG-3'.PrimerP2:F:5'-CACCGGCTACTATGGGAACT-3'; R: 5'-ACTTGCCACACCCTGGTC-3'.

Polymerase chain reactions were carried out in 25 µl volume containing approximately 2.0 µl of 10 µmol/l each primer, 2.5 µl of 10×PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.0), 0.1% Triton X-100), 1.5 µl of 20 mmol/l $MgCl_2$, 2.5 µl of 2.5 mmol/l each dNTP, 3.0 µl of 50 ng/µl genomic DNA, 1.0 µl of 2.5 U/µl *Taq* DNA polymerase (PROMEGA, Madison, WI, USA), made up with ddH_2O. Amplification conditions were as follows: initial denaturation at 94°C for 5 min; followed by 31 cycles of denaturation at 94°C for 30 s, annealing for 30 s (60.0°C for P1, 59.5°C for P2), extension at 72°C for 30 s with a final extension at 72°C for 8 min on Mastercycler® 5333 (EPPENDORF AG, Hamburg, Germany).

A volume of 2 µl PCR product was transferred in an Eppendorf tube, mixed with 7 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 centrifuged (G = 2000) and denatured at 98°C for 10 min, then chilled on ice for 8 min and loaded on 8% (primer P1) or 10% (primer P2) neutral polyacrylamide gels (acrylamide:bisacrylamide = 29:1). Electrophoresis was performed in 1×Tris borate (pH 8.3)-EDTA buffer at 9-15 V/cm at 4°C overnight. Then the gels were stained with silver nitrate to identify SSCP, photographed and analysed using an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (ALPHA INNOTECH CORP., San Leandro, CA, USA).

After SSCP analysis, PCR products of different homozygous genotypes were separated in 1% agarose gels and recovered using Geneclean kit (PROMEGA). The ligation reaction was conducted as per the instructions of the manufacturer (PROMEGA). Each DNA fragment was then transformed into *Escherichia coli* DH5α competence cell. Positive clones of transformed cells were identified by restriction enzyme digestion. Two clones of each genotype were selected and sequenced. Each clone was sequenced thrice. The target DNA fragments in recombinant plasmids were sequenced from both directions using an ABI3730 automatic sequencer (PERKIN ELMER APPLIED BIOSYSTEMS, Foster City, CA, USA) by Shanghai INVITROGEN BIOTECHNOLOGY Ltd., (Shanghai, China).

The following fixed effects model was employed for analysis of litter size in Jining Grey goat does and least squares mean was used for multiple comparison of litter size among different genotypes.

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

where:

y_{ijklm} – phenotypic value of litter size;

μ – population mean;

S – fixed effect of the i-th sire (i = 1, 2, 3, 4, 5);

KS_j – fixed effect of the j-th kidding season (j = 1, 2, 3, 4);

P_k – fixed effect of the k-th parity (k = 1, 2, 3);

G_l – fixed effect of the l-th genotype (l = 1, 2, 3), and

e_{ijklm} – 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 and discussion

Genomic DNA of three goat breeds was amplified using two pairs of primers for *INHBB* gene. PCR products were detected by running electrophoresis in 2% agarose gel (Fig. 1). The amplified products were consistent with the target fragments and had a good specificity which could be directly analysed by SSCP. Only the PCR products amplified by primer P2 displayed polymorphism. By P2 three genotypes (AA, AB and BB) were detected (Fig. 2).

Sequencing revealed one single nucleotide mutation (A→G) at base 782 of exon 2 of *INHBB* gene in BB genotype compared to AA genotype (Fig. 3).

Genotype and allele frequencies of *INHBB* gene in three goat breeds are presented in Table 1. For primer P2, three genotypes (AA, AB and BB) were detected in Jining Grey goats, and two (AB and BB) in Inner Mongolia Cashmere and Angora

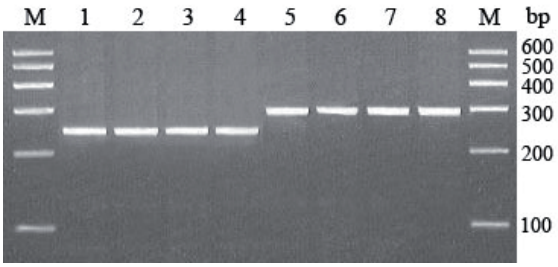

Fig. 1. PCR products of two pairs of primers.1-4: PCR products of P1; 5-8: PCR products of P2; M: SD002 marker.

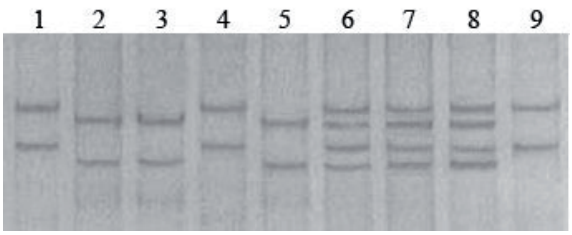

Fig. 2. SSCP analysis of PCR amplification using primer P2 in different goat breeds. 1, 4, 9 – AA genotype; 2, 3, 5 – BB genotype; 6, 7, 8 – AB genotype.

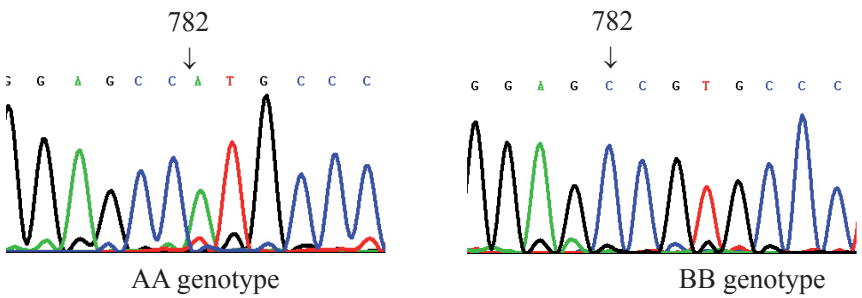

Fig. 3. Sequence comparison of AA and BB genotypes of primer P2 in goats.

goats. Jining Grey, Inner Mongolia Cashmere and Angora goats were in Hardy-Weinberg equilibrium (chi-square = 3.36, P=0,1861; 0.11, P=0.9461; 0.69, 0.7098, respectively). In the present study no homozygous AA does were identified in Inner Mongolia Cashmere or Angora goats. The reasons may be as follows: (i) A allele was rare; AA genotype frequency was so low that homozygous AA could not be detected in 40 Inner Mongolia Cashmere or 38 Angora goats. (ii) Homozygous AA does suffered from distinct developmental and reproductive defects, as well as from the embryonic death, or they were sifted out before arriving at reproductive age. Mating AB bucks to AB does, extensive sampling (larger goat samples of both sexes and more goat breeds), as well as DNA analysis would be required to verify this hypothesis. Such a study would have important implications for the goat industry.

Table 1. Genotype and allele frequencies of exon 2 of *INHBB* gene in three goat breeds

Frequency	Jining Grey goat (n=146)	Inner Mongolia Cashmere goat (n=40)	Angora goat (n=38)
Genotype			
AA	0.068 (10)	0.000 (0)	0.000 (0)
AB	0.500 (73)	0.100 (4)	0.237 (9)
BB	0.432 (63)	0.900 (36)	0.763 (29)
Allele			
A	0.318	0.050	0.118
B	0.682	0.950	0.882

Bracketed are numbers of does.

As inhibin plays an important role in FSH regulation and acts as a growing factor in ovary, it was proposed as a candidate gene for reproductive performance [Xue *et al*. 2004, Phillips 2005]. Jaeger and Hiendleder [1994] analysed 1000 lambing records and found that *INHBB* gene had obvious genetic effect on litter size of sheep. And, litter size was significantly influenced ($P<0.05$) by sire, kidding season, parity and *INHBB* genotype. The least squares means and standard errors for litter size of different genotypes of *INHBB* gene found in this study in Jining Grey goats are given in Table 2.

Table 2. Least squares means and standard errors for litter size of different genotypes of *INHBB* gene in Jining Grey

Genotype	No. of does	Litter size
AA	10	$2.76^{a}\pm0.21$
AB	73	$2.03^{b}\pm0.13$
BB	63	$1.92^{b}\pm0.16$

^{ab}Least squares means bearing different superscripts are significantly different at $P<0.05$.

The Jining Grey goat does with genotype AA had by 0.73 ($P<0.05$) or 0.84 ($P<0.05$) kids more than those with genotype AB or BB, while the does with genotype AB had by 0.11 ($P>0.05$) kids more than those with genotype BB. These results provide further evidence that inhibin is an important functional factor in ovary. The conclusion obtained in this study is preliminary because of low number of samples available. Further analyses are needed based upon more numerous animals and breeds.

REFERENCES

1. GOLDAMMER T., BRUNNER R.M., HIENDLEDER S., SCHWERIN M., 1995 − Comparative mapping of sheep inhibin subunits α (INHA) and β_B (INHBB) to chromosome 2 in goat by FISH. ***Mammalian Genome*** 6, 685-686.
2. JAEGER C., HIENDLEDER S., 1994 − Cosmid cloning and characterization of the coding regions and regulatory elements of the ovine α-(INHA), β_A-(INHBA) and β_B-inhibin (INHBB) genes. ***Animal Genetics*** 25, Supplement 2, p. 33.
3. LING N., YING S.Y., UENO N., ESCH F., DENOROY L., GUILLEMIN R., 1985 − Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. ***Proceedings of the National Academy of Sciences of the United States of America*** 82, 7217-7221.
4. MASON A.J., HAYFLICK J.S., LING N., ESCH F., UENO N., YING S.Y., GUILLEMIN R, NIALL H., SEEBURG P.H., 1985 − Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. ***Nature*** 318, 659-663.
5. MCNATTY K.P., HEATH D.A., HUDSON N.L., BALL K., CONDELL L., 1992 − Concentrations of immunoreactive inhibin in ovarian and peripheral venous plasma and follicular fluid of Booroola ewes that are homozygous carriers or non-carriers of the Fec^B gene. ***Journal of Reproduction and Fertility*** 95, 489-502.
6. PHILLIPS D.J., 2005 − Activins, inhibins and follistatins in the large domestic species. ***Domestic Animal Endocrinology*** 28, 1-16.
7. RIVIER J., SPIESS J., MCCLINTOCK R., VAUGHAN J., VALE W., 1985 − Purification and partial characterization of inhibin from porcine follicular fluid. ***Biochemical and Biophysical Research Communications*** 133, 120-127.
8. ROBERTS A.J., REEVES J.J., 1988 − Kidding rates of Angora goats passively immunized against estrogens. ***Journal of Animal Science*** 66, 2443-2447.
9. ROBERTSON D.M., FOULDS L.M., LEVERSHA L., MORGAN F.J., HEARN M.T., BURGER H.G. WETTENHALL R.E., de KRETSER D.M., 1985 − Isolation of inhibin from bovine follicular fluid. ***Biochemical and Biophysical Research Communications*** 126, 220-226.
10. RODGERS, R.J., STUCHBERY, S.J., FINDLAY, J.K., 1989 − Inhibin mRNAs in ovine and bovine ovarian follicles and corpora lutea throughout the estrous cycle and gestation. ***Molecular Cell Endocrinology*** 62, 95-101.
11. SAMBROOK, J., RUSSELL, D.W., 2001 − Molecular Cloning − A Laboratory Manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
12. THOMPSON, D.A., CRONIN, C.N., MARTIN, F., 1994 − Genomic cloning and sequence analyses of the bovine α-, β_A- and β_B-inhibin/activin genes. Identification of transcription factor AP-2-binding sites in the 5'-flanking regions by DNase I footprinting. ***European Journal of Biochemistry*** 226, 751-764.
13. TU, Y.R., 1989 − The Sheep and Goat Breeds in China, pp 88-90, 98-101, Shanghai Science and Technology Press, Shanghai, P.R. China. (in Chinese).
14. VASSALLI, A., MATZUK, M.M., GARDNER, H.A., LEE, K.F., JAENISCH, R., 1994 − Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. ***Genes and Development***. 8, 414-427.
15. WOODRUFF T.K., BESECKE, L.M., GROOME, N., DRAPER, L.B., SCHWARTZ, N.B., WEISS, J., 1996 − Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. ***Endocrinology*** 137, 5463-5467.
16. XUE, Y., CHU, M.X., ZHOU, Z.X., 2004 − Advances on inhibin genes. ***Hereditas*** (Beijing). 26, 749-755 (in Chinese, summary in English).