A new insertion/deletion fragment polymorphism of inhibin-α gene associated with follicular cysts in Large White sows

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ABSTRACT. Ovarian follicular cysts are anovulatory follicular structures that lead to infertility. Hormones play key roles in the formation and persistence of cysts. Inhibins are heterodimeric gonadal glycoprotein hormones that belong to the transforming growth factor-β superfamily. These hormones suppress the secretion of follicle-stimulating hormone. In this report, partial fragment of inhibin-α (INHA) subunit gene of Large White pig was detected from the genomic DNA by polymerase chain reaction. The sequence showed a 283 bp fragment insertion/deletion (I/D) polymorphism in INHA subunit gene. A total of 49 Large White sows with cystic follicles and 152 normal sows were screened for this polymorphism. The relationship of INHA I/D polymorphisms with follicular cysts was investigated. The distribution of I/D was significantly different between cystic and normal sows, thereby suggesting that the INHA subunit gene might be a potential biological marker for breeding programs in pig.

KEY WORDS: follicular cyst, inhibin α, polymorphism, swine

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Inhibins are heterodimeric gonadal glycoprotein hormones belonging to the growth factor-β superfamily. These hormones are called inhibins for their ability to suppress follicle-stimulating hormone (FSH) synthesis and secretion from anterior pituitary via negative feedback [10]. Inhibins are composed of a common inhibin-α subunit (INHA, 14 kDa) and one of two inhibin-β subunits (INHβA, 18 kDa; or INHβB, 18 kDa).

Previous studies have demonstrated that inhibins are associated with the balance of the endocrine in the hypothalamo-hypophyseal-gonadal axis [7] and are essential for animal reproduction. In females, ovaries are the main source of inhibins. Gonadotropes are the main target cells of inhibin [1]. Besides inhibiting FSH secretion via endocrine, inhibins also exert local action through autocrine and paracrine pathways in reproductive tissues [17]. These hormones play key roles in folliculogenesis, oocyte maturation and embryo development in females [16] by regulating granulosa cell proliferation and development. In in vitro cultured goose granulosa cells, in which inhibin-α subunit gene expression was down-regulated by RNAi, the apoptosis and proliferation indices were significantly higher than in the control groups; the G1 phase percentage decreased, and a corresponding increase in the S phase occurred [3]. Immunization against inhibin can increase the ovulation rate [13] and litter size [12] in domestic animals.

The important roles of INHA gene make it a strong candidate gene in mammalian reproduction. In this study, a new insertion/deletion (I/D) polymorphism of the pig INHA gene was detected by polymerase chain reaction (PCR). The relationship between the polymorphisms of INHA gene and follicular cysts was investigated in sows.

Ovaries from 49 Large White sows with cystic follicle and 152 normal sows in the age range of 5–6 months old were collected from a local slaughterhouse. The normal ovaries and follicular cysts were identified through the size of follicular diameter combined with the histological structure and hormone changes, as described in our previous research [16]. Cystic follicles were greater than 21 mm in diameter and were characterized by fluid-filled structures with a smooth and thin wall in the absence of corpus luteum. Genomic DNA was extracted from samples of ovary tissues (30 mg) by using a Multisource Genomic DNA Miniprep Kit (AXY-GEN, Hangzhou, China). The DNA quality and concentration were determined using a NanoDropTM ND-2000 UV-Vis spectrometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). Primers (F:5'-CCCCGTGTCCTCAGGATACCTAGT-3'; R:5'-GTGCTGGGACGGCGGAATAC-3') that amplified this 663 bp (or 380 bp) fragment of the partial intron containing 283 bp I/D fragment were designed according to the porcine INHA gene sequence (GenBank ID: 397386) using Primer 5.0. PCR was performed in reaction mixtures containing 100 ng DNA, 2.5 µl of 10× buffer, 5 pmol of each primer, 2 µl of 2.5 mM dNTPs and 2 units of pfu DNA polymerase (Promega, Madison, WI, U.S.A.) at a final volume of 25 µl. The conditions for amplification were as follows: 4 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 59°C, 30 sec at 72°C and finally, 10 min at 72°C. PCR products were identified on 1.5% agarose gel electrophoresis to confirm I/D variants based on their size differences (Fig. 1). A polymorphism with one fragment (663 bp in length) was characterized as I/I. A polymorphism

NOTE Theriogenology

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with one fragment of 380 bp length was characterized as D/D. A polymorphism with both fragments was characterized as I/D. Subsequently, PCR products with I/I and D/D fragments were amplified using pfu DNA polymerase (Promega), purified using Spin Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China), cloned into bacterial plasmid by using the pMD-18T vector (TaKaRa, Tokyo, Japan) and sequenced using ABI 3730X DNA sequence at the BGI (BGI, Tianjin, China).

According to the sequencing results, a fragment of I/D (283 bp in length) from nt 2495 to nt 2777 was present in Large White sows (Fig. 2). Numbering is scored relative to the first nucleotide (+1) of the start codon in the sequencer or analyzer of the INHA gene in pig. This I/D polymorphism was located within the intron.

Hardy–Weinberg equilibrium for genotypic distribution was investigated by using the Chi-Square test. A deviation from the Hardy–Weinberg equilibrium for the INHA I/D polymorphism was observed in the normal and cystic groups. Genotype and allele frequencies between cystic and normal subjects were compared by Pearson’s Chi-Square test, and the results are summarized in Table 1. The genotype distribution of the I/D polymorphism in the cystic groups was significantly different from those in the normal group. The II, ID and DD rates for the cystic and normal groups (P=0.03) were 63.3%, 20.4% and 16.3% and 45.4%, 17.8% and 36.8%, respectively. The allele I frequency in cystic groups was higher than in the normal group (73.5% vs. 54.3%).

The distribution of the I/D polymorphism in the cystic groups was significantly lower than in normal large follicles. Thus, the ability to downregulate the FSH level through negative feedback was affected. Furthermore, INHA also exerts local action in folliculogenesis by regulating the granulosa cell proliferation and apoptosis through the autocrine and paracrine pathways [16, 17]. In cystic follicles, the expression level of β-glycan, which is the co-receptor of inhibin, was significantly lower than in normal follicles [21]. These results indicated that the inhibin-β-glycan signaling pathway was disrupted in cystic follicles.

Other studies suggest that ovarian cysts should be classified as a quantitative trait disorder [15]. The INHA gene was identified in one of the quantitative trait loci for ovulation rate in pig [10]. Linkage disequilibrium may occur between
Fig. 2. Sequences of the deletion fragment of the INHA gene of pig. Upper line was the sequence of the gene of INHA published on NCBI (GenBank: No. 397386). Numbering is scored relative to first nucleotide (+1) of start codon in sequence of INHA gene in pig; Lower line was the sequence of the PCR product of D/D fragments.

Table 1. Frequency distribution of INHA I/D alleles and genotypes in cystic sows and normal group No. (%)

| Variables | All sows (n=201) | Cystic (n=49) | Normal (n=152) | P value | OR (95 CI) |
|-----------|-----------------|---------------|----------------|---------|------------|
| II        | 100 (49.8)      | 31 (63.3)     | 69 (45.4)      | 0.03    | 0.483 (0.249–0.937) |
| ID        | 37 (18.4)       | 10 (20.4)     | 27 (17.8)      |         |            |
| DD        | 64 (31.8)       | 8 (16.3)      | 56 (36.8)      |         |            |
| I         | 237 (59.0)      | 72 (73.5)     | 165 (54.3)     | 0.001   | 0.429 (0.259–0.708) |
| D         | 165 (41.0)      | 26 (26.5)     | 139 (45.7)     |         |            |

P value assessed using Pearson’s chi-square test (two-tailed) compared with normal group. OR: odds ratio; CI: 95% confidence interval. a) Recessive genetic model: II versus I/D+DD.
the INHA gene mutations detected in this study and other genes located near the INHA loci.

In summary, we identified a new I/D of INHA polymorphism in pig by using PCR. I/D polymorphism is significantly associated with the presence of follicular cysts. Sows with I allele have a higher risk of developing follicular cysts. These findings may provide a novel biological marker and promising genetic therapy candidates for ovarian cysts in pigs, which would greatly benefit pig breeding programs. However, ovarian cysts may be induced by other factors, such as age, nutrition, and season of the year [6, 11], and some cysts may disappear approximately 1 week after formation [2]. More studies are needed to confirm the relationship between the formation of ovarian cysts and genetic markers in other breeds.

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