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RELATIONSHIP BETWEEN THE INSERTION/DELETION VARIANTS OF *POU1F1*, *FSHB*, AND*MUC13* AND TESTIS MEASUREMENT TRAITS IN MALE PIGLETS

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ABSTRACT

The aim of this study was to investigate the insertion/deletion (indel) mutations of the POU domain class 1 transcription factor 1 (*POU1F1*), the follicle-stimulating hormone β-subunit (*FSHβ*), and themucin 13 (*MUC13*) genes, as well as to evaluate their associations with testis measurement traits in male piglets. In total, these indels were analyzed in 442 individuals from two pig breeds by PCR and agarose gel electrophoresis. As a result, three genotypes (homozygous insertion and heterozygote and homozygous deletion) were found at each locus. Association analyses revealed a significant relationship between *POU1F1* indel and testis short girth (TSG) in 15-day-old Large White (LW) pigs (P = 0.016). At the *POU1F1*indel locus, the testis measurement traits of pigs with the AA and AB genotypes were larger than those of pigs with the BB genotype. As for *FSHβ*, an indel was found to be significantly associated with testis long circumference (TLC) (P = 0.050) and testis weight (TW) (P = 0.001) of 40-day-old Landrace (LD) piglets, with the BB genotype showing the largest testis measurement traits. We found no significant relationships between indels of *MUC13* and the testis measurement traits. The significant influence of indels of*POU1F1* and *FSHβ* suggests that both *POU1F1* and *FSHβ* influence reproductive potential, and therefore, could be possible candidate genes for the breeding improvement of male piglets.

Keywords: Pigs; *POU1F1* gene; *MUC13* gene; *FSHβ* gene; Insertion/deletion (indel).

Abbreviation: indel, insertion/deletion; LW, Large White; LD, Landrace; MAS, marker-assisted selection; SNPs, single nucleotide polymorphisms;POU1F1, POU domain, class 1, transcription factor 1;RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; FSH, Follicle-stimulating hormone; MUC, Mucins; TW, testis weight; TLC, testis long circumference; TSG, testis short girth;TD-PCR, touchdown PCR;HWE, Hardy-Weinberg equilibrium; PIC, Polymorphism information content; ANOVA, analysis of variance; GLM, general linear model;Ho, homozygosity; He, heterozygosity; Ne, effective allele numbers; GH, growth hormone; PRL,prolactin.

INTRODUCTION

In the past two decades, there has been an increasing interest in understanding the genetic determinants of reproductive traits in livestock. This has led to improvement in the global pig industry, an economically important livestock for agriculture (Park *et al*., 2015). Research toward improving reproductive traits is considered more promising than feeding and housing improvements (Buske, 2006). Hence, there is major interest among breeders to identify reproductive traits that can be selected.

Various marker-assisted selection (MAS) programs have been developed in order to increase the economic performance of swine farms (Marantidis *et al*., 2016). In particular, identification of insertion/deletion (indel) events has allowed the identification of relevant pig genes to a greater extent than the study of single nucleotide polymorphisms (SNPs) (Sathya *et al*., 2014). For example, the 18-bp indel of *SOX9* has been identified as one of the functional genes for porcine inguinal and/or scrotal hernia (Brenig *et al*., 2015). To date, however, there have been no studies regarding candidate genes directly related to reproduction such as those affecting male pigs testis size.

POU domain class 1 transcription factor 1 (*POU1F1*) is the first pituitary-specific transcription factor to be identified in the human and mouse (Wang *et al*., 2015). The pig*POU1F1* gene is located on chromosome 13 and contains 6 exons and 5 introns. Its partial genomic sequence has been reported (Yu *et al*., 2001). *POU1F1* contains a POU DNA-binding domain with two regions: a POU homeodomain required for DNA binding and a POU-specific domain essential for DNA-binding specificity and dimerization (Ozmen *et al*., 2014). The POU1F1 transcription factor is expressed in the pituitary gland where it regulates pituitary development and the expression of growth hormone genes (Cohen *et al*., 1996). Previous research revealed genetic polymorphisms in *POU1F1* by using the POU

domain probe, restriction fragment length polymorphism (RFLP), and polymerase chain reaction (PCR) techniques (Sadeghi *et al*., 2014). A significant genotype effect of *POU1F1* on average daily weight gain was found in the finishing period when focusing on crossbred 45, 70, and 180-day pig populations (Song *et al*., 2005). Therefore, *POU1F1* is an essential factor regulating the development and reproduction of animals (Wu *et al*., 2009). Several indels including one potentially important 313-bp indel have been identified in Chinese pigs (Song *et al*., 2007). However, to date no studies have examined the association between testis measurement traits and *POU1F1*indels in male piglets.

Follicle-stimulating hormone (FSH) is a member of the pituitary glycoprotein hormone family (Kato *et al*., 2011). Each hormone in the family shares a common α-glycoprotein subunit and contains a unique β-subunit that confers physiological specificity to the respective hormone (Aikawa*et al*., 2004). Both subunits of FSH participate in receptor binding and signaling effects, but each has its own function. The effect of *FSH* on animal reproductive performance is mainly affected by genetic variation within the β-subunit, both in its gene regulatory region and its coding region. A 280-bp indel of *FSHβ* has been confirmed in different pig breeds(Zhao *et al*., 1999). To date, however, the possible functional significance of this 280-bp indel of *FSHβ* is unknown in pigs.

Mucins (MUC) are glycoproteins that cover the apical surfaces of epithelial cells in gastrointestinal and respiratory tracts, forming the first line of host defense against enteric pathogens (Zhang *et al*., 2008). The protein family mediates interactions between epithelial cells and their milieu by modulating cell adhesion, the lubrication and protection of mucosa, the renewal and differentiation of epithelia and cell signal transduction (RingelandLöhr, 2003). Previous studies have shown that *MUC* is in close linkage disequilibrium with the F4bcR locus, which acts as a marker to identify susceptible pigs (Fontanesi *et al*., 2012). *MUC13* is a transmembranemucin that is highly expressed in the gastrointestinal tract (Williams *et al*., 2001). The aberrant expression of MUC13 is associated with intestinal-type gastric cancer, colorectal cancer and inflammatory bowel disease (Moehle *et al*., 2006). The 68-bp indel mutation in intron 2 of *MUC13* has been reported as an important anti-disease genetic marker for molecular breeding in the Yorkshire population(Sun *et al*., 2015).MUC13 aslo is one of hormonal control of mucin proteins (Poon*et al*., 2014). However, until now, the 68-bp indel of *MUC13* and its influence on the testis measurement traits in male piglets is unknown.

In some mammals, testis measurement traitscan reflect spermatogenesis (Gouletsou *et al*., 2008). Hence, identifying individual differences in testis measurement traits may be key to selecting animals with high reproductive capacity. Some studies focusing on rams and bulls reported that testis measurement traits might be an indicator of fertility, suggesting high correlation between the traits (Devkota *et al*., 2008; Rege *et al*., 2000). Chen *et al*. (2016) from our group found that an indel in the sperm flagella 2 (*SPEF2*) gene on chromosome 16 could influence the testis measurement traits of boars (Chen *et al*., 2016).

Related studies have identified a number of different indels in *POU1F1*, *FSHβ*, and *MUC13*. The purpose of this study was to investigate the indels of *POU1F1*, *FSHβ*, and *MUC13* in the Large White (LW) and Landrace (LD) pigs, two of the most popular breeds in many countries, including China (Bergfelder-Drüing *et al*., 2015), and to evaluate their association with testis measurement traits in male piglets for the first time. Our findings would contribute toward improving the breeding and genetics in male piglets.

MATERIALS AND METHODS

Animal sources and data collection (testicular traits): A total of 442fresh and complete testis samples were obtained from castrated male piglets belonging to two breeds, LD and LW, which were reared at the National Swine Breeding Farm, Ankang, Shaanxi, China. Among of them, all LD male piglets (n=72) were 40 days old; 32.43% LW piglets (n=120) were 40 days old, while 67.57% LW piglets (n=250) were 15 days old. Data on testis weight (TW), testis long circumference (TLC) and testis short girth (TSG) were obtained from the testicular tissues, to be used for association evaluation analyses (Chen *et al*., 2016).

Genomic DNA isolation and DNA pool construction: Genomic DNA of the above samples was isolated from the testis samples following the procedure as described in Sambrock and Russell (Sambrook and Russell, 2002) and Lan *et al* (Lan *et al*., 2007). Those DNA samples were quantified and subsequently diluted to 50 ng/μL as their working solutions (Wu *et al*., 2014). DNA pools were constructed with 50 individual genomic DNA samples randomly chosen from each pig breed and used as a PCR template (Shi *et al*., 2016).

Primer design and PCR amplification: Three pairs of PCR primers for *POU1F1*,*FSHβ*, and *MUC13* were designed according to Song *et al*. (Song *et al*., 2007), Zhao *et al*. (Zhao *et al*., 1999), and Sun *et al*. (Sun *et al*., 2015) (Table 1), respectively. All PCR primers were synthesized by GenScript (Nanjing, Jiangsu Province, China). The PCRs of *POU1F1*, *FSHβ* and *MUC13* were performed in a volume of 20 μL, which contained 1 μL of template DNA, 0.5 μL of each primer (forward and reverse primer), 10 μL Primer Script RT Enzyme Mix I and 8 μL ddH_2O.All PCR reactions were carried out using the touchdown PCR (TD-PCR) procedure and products detected by agarose gelelectrophoresis.TD-PCR

reactions were carried out as follows: initial denaturation for 4 min at 95 °C, followed by 15 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 65 °C (with a decrease of 1 °C per cycle), and extension for 1000 bp/min at 72 °C, another 23 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1000 bp/min at 72°C, and a final extension of 10 min at 72 °C.

Statistical analysis: The Hardy-Weinberg equilibrium (HWE), genotypic and allelic frequencies were directly calculated using the SHEsis program (http://analysis.bio-x.cn) (Shi *et al*., 2016). Polymorphism information content (*PIC*) was calculated using an online calculator (http://www.msrcall.com/Gdicall.aspx) (Wu *et al*., 2014; Jia *et al*., 2015; Yang *et al*., 2016). The associations test of the indels with three testis measurement traits (TW, TLC, and TSG) were considered at two different growth periods (15-day/40-day) in LW piglets, and one period (40-day) in LD piglets, respectively. These association analyses were performed by analysis of variance (ANOVA) on the software SPSS (Version 18.0) if data agreed with the characteristics of normality and homogeneity of variances. If not, the nonparametric test (Kruskal–Wallis) was conducted using SPSS (18.0). The ANOVA applied the general linear model (GLM) and the reduced linear model was as follows: $Y_{ijk}=\mu+\alpha_i+\beta_j+\varepsilon_{ijk}$, where Y_{ijk} represents the observation of the testis measurement traits (TW, TLC, and TSG) evaluated on the i^{th}level of the fixed factor age (α_i) and the j^{th} level of the fixed factor genotype or combined genotype (β_j); μ represents the overall mean for each trait; and ε_{ijk} is the random error for the ijk^{th} individual (Pan *et al*., 2013). Theassociated LD and LW breed effect was not included in the linear model, as an initial statistical analysis indicated that the effect did not have a significant influence on the variability of traits in the pigs (Chen *et al*., 2016).

RESULTS

The frequency of the indel variants: In this study, the indel variants of *POU1F1*, *FSHβ*, and *MUC13* were investigated using polyacrylamide gel electrophoresis analysis.

As shown in Figure 1, the electrophoretogram revealed three genotypes (named AA, BB, and AB) for *POU1F1* (Fig.1a), *FSHβ* (Fig.1b), and *MUC13*(Fig.1c), respectively. In the analysis of *POU1F1*, the genotype BB exhibited one band (1926 bp), the AA genotype exhibited one band (2239 bp), and the AB genotype exhibited two bands (2239 bp and 1926bp) (Fig.1a). *FSHβ* showed a homozygote insertion type (AA) consisting of 500 bp, a deletion type (BB) consisting of 220 bp and a heterozygote type (AB, 500 bp and 220 bp) (Fig.1b). *MUC13* also showed three genotypes: AA (151 bp), BB (83 bp), and AB (151 bp, 83 bp) (Fig.1c).

The frequencies of alleles of the three genes were analyzed (Table 2). Our results indicated that the AA genotype of *POU1F1* had a high frequency in LD and LW breeds. Furthermore,the B allelic frequencies in LD and LW breeds, respectively, were 0.902 and 0.664 for *FSHβ* and 0.825 and 0.580 for *MUC13*. In order to evaluate genetic diversity, we calculated homozygosity (*Ho*), heterozygosity (*He*), effective allele numbers (*Ne*), and *PIC* at each locus (Table 2). The *PIC* value showed that the LW breed possesses medium genetic diversity ($0.25 < PIC < 0.5$), whereas the LD breed has low genetic diversity ($0 < PIC < 0.25$) in the indel locus. The χ^2 test showed that the genotypic frequencies of *POU1F1*, *FSHβ*, and *MUC13* are in agreement with the HWE ($P > 0.05$) (Table 2).

Associations between the indel variants and the testis measurement traits in male piglets: In the two breeds, the associations between the different indels of three genes and the pig testis measurement traits were investigated (Table 3). A significant relationship was observed between the 313-bp indel of *POU1F1* and TSG at 15-day-old in LW pigs ($P = 0.016$). The BB genotypes showed a lower TW than other genotypes. Furthermore, significant relationships were observed between the 280-bp indel of *FSHβ* and testis measurement traits (TLC and TW) in the 40-day old LD breed($P = 0.050$ and $P = 0.001$, respectively). At the 40-day old stage, LD individuals with *FSHβ*genotype BB showed greater testis measurement traits than those with genotypes AA and AB. However, we found no significant relationships between the 68-bp indel of *MUC13*and testis measurement traits.

Table 1. Amplification PCR primer sequences of the pig *POU1F1*, *FSHβ* and *MUC13* genes.

Primer	Primer sequences (5′–3′)	Sizes (bp)	Detection	Notes
POU1F1	F:ATAGGTTGGGATGAGAAGAAT R:GGTTTCCATAATGACAGGAAGGG	2239/ 1926	AA=2239 bp AB=2239 bp +1926 bp BB= 1926bp	Cited from Song *et al*. (2007)
FSHβ	F:CCTTTAAGACAGTCAATGGC R:ACTGGTCTATTCATCCTCTC	500/ 220	AA=500 bp AB=500 bp +220 bp BB=220 bp	Cited from zhao *et al* (1999)
MUC13	F:TTCTACTCTGATTCCACATCACG R:TGGTCATGTCTAGGACTCTTTGAG	151/ 83	AA=151bp AB=151 bp +83 bp BB=83 bp	Cited from Sun *et al* (2015)

Table 2 Genotypic and allelic frequencies and population indexes for pig *POU1F1*, *FSHβ* and *MUC13* genes.

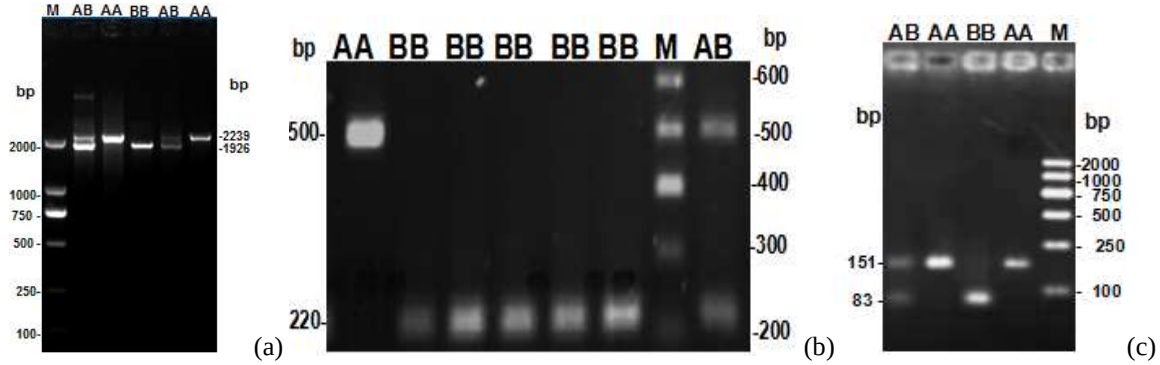
Breeds/Loci	Sizes	Genotypic frequencies			Allelic frequencies		HWE	Population parameters			
POU1F1-indel	N	BB	AB	AA	B	A	P values	Ho	He	Ne	PIC
LD	72	0.000	0.130	0.870	0.065	0.935	P>0.05	0.879	0.121	1.138	0.114
LW	372	0.059	0.364	0.577	0.241	0.759	P>0.05	0.634	0.366	1.576	0.299
FSH-β-indel	N	BB	AB	AA	B	A	P values	Ho	He	Ne	PIC
LD	72	0.459	0.410	0.131	0.664	0.336	P>0.05	0.554	0.446	1.806	0.347
LW	372	0.818	0.167	0.014	0.902	0.098	P>0.05	0.823	0.177	1.215	0.161
MUC13-indel	N	BB	AB	AA	B	A	P values	Ho	He	Ne	PIC
LD	72	0.683	0.286	0.032	0.825	0.175	P>0.05	0.712	0.288	1.405	0.247
LW	372	0.315	0.537	0.154	0.580	0.420	P>0.05	0.513	0.487	1.950	0.368

Note: HWE, Hardy-Weinberg equilibrium; Ho, homozygosity; He, heterozygosity;Ne, effective allele numbers; PIC, Polymorphism information content.

Table 3. Relationship between the indels and reproduction traits in male piglets (LSM±SE)(*P*<0.05).

Genes	Breeds	Traits	Genotypes			p
			AA	AB	BB	
POU1F1	15days Large white	TSG(cm)	4.27±0.09^{a}	4.16±0.15^{a}	2.20±0.00^{b}	0.016
FSHβ	40 days Landrace	TLC(cm)	6.64±0.15^{b}	6.99±0.12^{ab}	7.09±0.11^{a}	0.050
	breeds	TW(g)	5.86±0.30^{c}	6.03±0.25^{ab}	7.50±0.32^{a}	0.001

Note: Cells with different letters (a, b) differed significantly (*P*<0.05); (a, c) differed extremely significantly (*P*<0.01).



(a) (b) (c)

Figure1. Electrophoresis pattern of the indel variants of *POU1F1*(a), *FSHβ*(b),and *MUC13*(c) genes in male piglets.

DISCUSSION

Reproduction traits constitute a major factor affecting productivity in the pig industry. It is well known that genetic improvements and enhanced management can further promote reproduction traits. To date, several studies have revealed many candidate genes related to reproduction in pigs (Bieniek-Kobuszewska *et al*., 2016; Chen *et al*., 2016). Previous studies reported that *POU1F1*, *FSHβ*, and *MUC13* had the potential to be significantly associated withproduction traits in pigs; however, their relation to testis measurement traits remained unknown. Indel had been used as high-throughput genetic markers in genetic analysis and marker-assisted breeding (Zang *et al*., 2016).To date, however, few studies had examined whether indels were significantly associated with testis measurement traits in pigs. Given this, the present study's goal was to evaluate the associations between indels in these genes and testis measurement traits in LW and LD pigs.

Many studies indicated that the pituitary hormones, including growth hormone (GH) and prolactin (PRL), play important roles in regulating pig development and reproduction, while *POU1F1* regulates the expression of GH and PRL by binding to target DNA promoters as a dimer (Jacobson *et al*., 1997). Thus, *POU1F1*was regarded as a potential candidate gene for reproduction and expected to be applied with marker-assisted breeding (Wu *et al*., 2009). Kim *et al* found that the SNPs of the *POU1F1* intron region 1 affect growth traits in pigs (Kim *et al*., 2014). In the current study, the 313-bp indel of *POU1F1* was identified by DNA pool sequencing and aligned with the sequence in the study by Song *et al*. (Song *et al*., 2007).It is notable that the genotype BB was inferior in the testis measurement traits when compared to other genotypes (AA and AB), suggesting that the allele A of the LW pig *POU1F1*imparts positive effects on the testis measurement traits in this breed. Interestingly, Song *et al* also found that the AA genotype of *POU1F1* was positively associated with birth weight in pigs (Song *et al*., 2007). These findings suggested that the indel polymorphism of *POU1F1*might be a potential DNA marker for male piglets.

In recent years, porcine FSH, a glycoprotein secreted by the anterior pituitary, has been deemed one of the candidate genes of porcine reproductive performance (Linville *et al*., 2001). Previous research had shown that *FSHβ* affected male reproductive performance in pigs (Xu *et al*., 2016). Luoreng *et al*. found that the 273-bp indel of *FSHβ* affects reproduction traits in the Beijing Black Pig (Luoreng *et al*., 2007). The present study focused on the indel polymorphism of *FSHβ*and its association with testis measurement traits in male piglets. Individuals with BB genotype had a better phenotype compared to those with AA and AB genotypes, suggesting that allele B of the pig *FSHβ*conferred positive effects on testis measurement traits. Genotype BB was also positively correlated to the number of pig births reported by Zhao *et al*. (Zhao *et al*., 1999). These findings suggested that the indel polymorphism of *FSHβ* might be a potential DNA marker for male piglets.

However, we found no significant relationshipbetween the indel polymorphism of *MUC13* and testis measurement traits. It was supposed to be the result of the differences between the multitudinous genetic backgrounds of LD and LW breed (Bressan *et al*., 2016). The lack or relationship may be because of *MUC13*just is a good candidate gene for improve breeding of diarrhea resistance in pigs, but not affect in testis measurement traits (Ren *et al*., 2012). While the results concerning *POU1F1*and *FSHβ*indels are exciting, further research is needed to reveal the molecular mechanisms through which these mutations affect male reproductive traits.

In conclusion, we found that the indels of *POU1F1* and *FSHβ*were significantly related to testis measurement traits. Our results suggest that these genes are potential DNA markers that may be applied in the improvement of reproductive performance and genetics in male pigs.

Conflict of interest: We confirm that this manuscript has not been published in whole or in part and is not being considered for publication elsewhere. There are no any ethical conflicts of interest for all authors. The corresponding authors, Dr. C.Y.Pan and X.Z.Sun, take responsibility on behalf of all authors for the authorship, authenticity and integrity of this manuscript, and affirms that all authors and acknowledged contributors have read and approved this manuscript.

Author contributions: F. Ren, C.Y. Pan and X.Z. Sun designed the study and wrote the paper. F. Ren, S. Yu, X.L. Zhang, X.Y. Lvperformed the experiments. F. Ren analyzed the data; X.Y. Lvcollected the samples; Dr.C.Y. Pan and X.Z. Sun edited and reviewed the manuscript.

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