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Polymorphisms of *Zygote arrest 1* gene and their effects on litter size in pigs*

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Zygote arrest 1 (ZAR1) is oocyte-specific protein involved in the initiation of embryo development. The objectives of this study were to identify novel mutations in *ZAR1* gene and investigate the association between its genetic variants and litter size in Chinese local and European pigs. A novel single nucleotide polymorphism (SNP) in exon 3 (g.2612T>C) and a 5 bp deletion/insertion in intron 3 (g.3838-3839insTGCAG) were found by sequencing and then their genotypes were identified in 218 sows of five breeds. The results of association analysis showed that the SNP g.2612T>C was significantly related to litter size in Durocs (P<0.05), the sows with genotype *CC* genotype gave more piglets than those of *TT* or/and *TC* genotypes. What's more, the litter size in the Duroc breed was also significantly dependent (P<0.05) on polymorphism in intron3 (g.3838-3839 sTGCAG). Sows with *NN* genotype had more piglets per litter than those of *MM* or/and *MN* genotypes. A similar relation (though not significant) was observed in remaining breeds tested. It is concluded that *ZAR1* gene might be a potential important candidate gene related to litter size in pigs.

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The oocyte-to-embryo transition depends on maternal transcripts and proteins that accumulate during oogenesis; maternal-effect genes have decided the embryonic development destiny [Latham 1999, 2001]. Oocytes are critical for post-ovulation events, such as fertilization, activation of the zygotic genome, and initiation of the early embryonic development. Pre-implantation embryo development is dependent on stored maternal factors [Zhao *et al*. 2006].

Zygote arrest 1 (*ZAR1*) is the first identified and one of the few known genes of oocyte-specific maternal-effect genes, which plays an important role in the oocyte-to-embryo transition and is essential for the beginning of embryo development. Wu *et al.* (2003a) found that *Zar1*-null (*Zar1*–/–) female mice were infertile, though *Zar1*–/– mice were viable and grossly normal. What's more, most embryos from *Zar1*–/– females were arrested at early stage of development, and their maternal and paternal genomes remained separate. Wu *et al*. [2003b] proposed that the carboxyl-termini of ZAR1 protein contained an important functional domain that was conserved through vertebrate evolution and that may be necessary for normal female reproduction in the transition from oocyte to embryonic life. In pigs, *ZAR1* gene was mapped to chromosome 8 p21-p23. At the amino acids level, identity of sequence reached 78% between pig and *Bos taurus* and 65% between *Homo sapiens* and pig. Expression of the porcine *ZAR1* gene has been documented exclusively in oocytes, persisted during the first cleavages in embryos developed *in vivo,* and declined rapidly in morulae and blastocysts [Uzbekova *et al*. 2006]. This gene is evolutionary conserved in vertebrates and ZAR1 protein is characterized by the presence of atypical plant homeobox zinc finger domain, suggesting its role in transcription regulation [Uzbekova *et al*. 2006]. So it is possible that the *ZAR1* gene has a significant effect on reproductive performance of pigs.

Reproductive performance, such as litter size, is important economic trait of pigs. Therefore, the objectives of this study are to identify mutations of *ZAR1* gene and analyse their effects on litter size in sows.

Material and methods

Animals and sampling

Blood samples were withdrawn from 218 sows aged 2.5 to 5 years of two Chinese local breeds (40 Small Meishan and 28 Qingping sows) and three European breeds (59 Duroc, 40 Landrace and 51 Large White sows). Small Meishan and Qingping sows were housed in the Conservation Center of Small Meishan Boars of Jiangsu province and the National Conservation Center of Qingping Boars, respectively. Duroc, Landrace and Large White were owned by the Hubei Tianzhong Boar Co., Ltd, China.

The genome DNA was extracted from peripheral blood leukocytes by standard procedure [Sambrook *et al*. 1989] and the concentrations of DNA samples were measured by both spectrophotometry (at 260 nm and 280 nm absorbance) and

electrophoresis (in 0.8% agarose gel stained with ethidium bromide for visualization under UV light). All samples were stored at -20°C.

The reproductive performance (total number of piglets born – TNB) and number of piglets born alive – NBA) were recorded, including TNB1 and TNB2 (total number of piglets born iin first and second litter, respectively), TNB3 (total number of piglets born in the third and later litters), NBA1 and NBA2 (number of piglets born alive in the first and second litter), NBA3 (number of piglets born alive in the third and later litters).

Identification of polymorphisms of *ZAR1* gene

The PCR (polymerase chain reaction) primers showed in Table 1 were designed using Primer Premier 5.0 based on *Sus scrofa ZAR1* gene (DQ231443) sequence. The following region of *ZAR1* gene was amplified using P_1 primers: partial intron 1– exon 2 – intron 2 – exon 3 – partial intron 3, whereas P_2 primers allowed the amplification of the region of *ZAR1* gene comprising partial intron 3 and exon 4.

Table 1. Primers for porcine *ZAR1* gene and related information

Primer	Primer Sequence (5'-3')	Tm (°C)	Product size (bp)
ZAR1 P_{1}-L	AAACACCTGCTTGGTAATCT	57	1264
ZAR1 P_{1}-R	AACTTGGGTATGTGCTTCAT		
ZAR1 P_{2}-L	TCGTCCTGTCCCTGTCGTT	59	396
ZAR1 P_{2}-R	GGCTCACTTGTCTGCTCCCT		

PCR products were sequenced in order to find the gene mutations – Lee *et al*. [1992] and Yuan *et al.* [2007a]. Four DNA samples selected randomly from each breed were used for sequencing by an ABI 377 automated sequencer (GMI, Inc., Ramsey, MN, USA) to identify the mutation. The sequencer software (dnastar5.0) was used to assembly the sequences and identify polymorphisms.

Genotyping

218 sows were genotyped by PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism). Two PCR amplicons, containing the g.2612T>C and g.3838_3839insTGCAG, respectively, were digested with specific restriction enzymes [BstU I (MBI FERMENTAS) and TspR I (MBI FERMENTAS), respectively] and then separated by 1.2% agarose gel electrophoresis and applying final staining with ethidium bromide.

Statistical

Chi-square analyses and Fisher's exact test (two sided) were performed in Statistical Package for the Social Sciences (SPSS version 16.0, SPSS Inc, Chicago, IL, USA) and Statistics Analysis System (SAS version 8.0, Cary, North Carolina, USA). Significance was established at $P<0.05$, and highly significant difference considered

at P<0.01. Association analyses were carried out using the models as below according to the protocol of Yuan *et al*. [2007b].

TNB records: $Y_{ij} = \mu+X_i + W_j+XW_{ij} +e_{ij}$;

NBA records: $Y_{ijk} =\mu+X_i + W_j +S_k+F_l+ XW_{ij} +XF_{il}+XS_{ik}+WF_{il}+XWFS_{ijkl}+e_{ijkl}$, where:

Y – observed trait;

μ – population mean;

X – fixed effects of genotype;

W – fixed effects of breed;

S – fixed effects of sire term;

F – fixed effects of litter;

XW – fixed interaction effects of genotype x breed;

XF – fixed interaction effects of genotype x litter;

XS – fixed interaction effects of genotype x sire term;

WF – fixed interaction effects of breed number x litter;

XWFS – fixed interaction effects of genotype x breed x sire term x litter;

e – random effects.

Results and discussion

Two mutations in the porcine *ZAR1* gene, single nucleotide polymorphism (SNP) in exon 3 (g.2612T>C) and a 5bp-deletion/insertion in intron 3 (g.3838_3839insTGCAG), were found by sequencing and genotyped through PCR-RFLP with the BstU I or TspR I restriction enzyme, respectively. Three genotypes at g.2612T>C were identified after digesting the 1264 bp PCR product with BstU I: genotype *TT* had only 1264 bp fragment, *CC* gave 768 bp and 496 bp fragments, and *TC* gave 1264 bp, 768 bp and 496 bp fragments (Fig. 1). Three genotypes at g.3838_3839insTGCAG were also detected after digesting the 396 bp PCR product with TspR I: genotype *MM* had 391 bp and 5bp fragments, *NN* had 311 bp, 80 bp and 5 bp fragments, and *MN* had 391 bp, 311 bp, 80 bp and 5 bp fragments (Fig. 2). In addition, the 5 bp fragment was out of Figure 2.

The allele and genotype frequencies of the two mutations are shown in Tables 2 and 3. For g.2612T>C *locus*, three genotypes were found in European breeds (Duroc, Landrace and Large White). However, only one genotype (*CC*) was detected in Chinese breeds (Small Meishan and Qinping). There were three genotypes in all five breeds at the mutation of g.3838_3839insTGCAG, and the frequency of allele *M* was higher than that of allele *N*. Furthermore, the frequency of *MM* genotype was highest

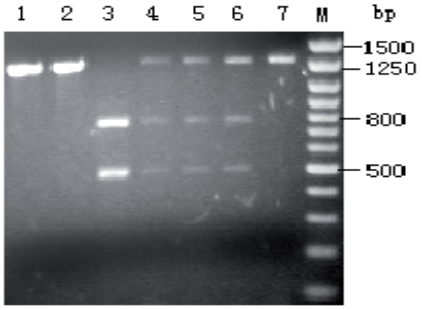

Fig. 1. Genotyping results of g.2612T>C at exon3 of ZAR1 gene. Lanes 1, 2 and 7 were TT genotype; lane 3 was CC genotype; lanes 4, 5 and 6 were CT genotype; and lane M was DNA marker.

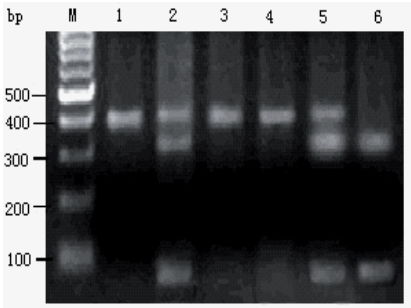

Fig. 2. Genotyping results of g.3838_3839insTGCAG at intron3 of ZAR1 gene. Lanes 1, 3 and 4 were MM genotype; lanes 2 and 5 were MN genotype; lane 6 was NN genotype; and lane M was DNA marker.

Table 2. Allele and genotype frequencies at g.2612T>C in pigs of five breeds

Breed	Genotype frequency			Allele frequency	
	CC	CT	TT	C	T
Small Meishan	1.000(40)	0(0)	0(0)	1.000	0
Qingping	1.000(28)	0(0)	0(0)	1.000	0
Duroc	0.136(8)	0.407(24)	0.457(27)	0.340	0.660
Landrace	0.575(23)	0.325(13)	0.100(4)	0.738	0.262
Large White	0.765(39)	0.176(9)	0.069(3)	0.853	0.147

Table 3. Allele and genotype frequencies at g.3838_3839insTGCAG in pigs of five breeds

Breed	Genotype frequency			Allele frequency	
	MM	MN	NN	M	N
Small Meishan	0.625(25)	0.275 (11)	0.100 (4)	0.763	0.237
Qingping	0.741(20)	0.037 (1)	0.222 (6)	0.760	0.240
Duroc	0.300(15)	0.520(26)	0.180(9)	0.560	0.440
Landrace	0.179(5)	0.679(19)	0.142(4)	0.519	0.481
Large White	0.441(15)	0.471(16)	0.088(3)	0.677	0.323

in Small Meishan and Qingping breeds, while the number of sows with *MN* genotype was greatest in the three European breeds.

For the SNP g.2612T>C, the association analysis was only performed in three European breeds due to only one genotype in two Chinese breeds. The g.2612T>C genotype was significantly associated with TNB1 ($P<0.05$), TNB2 ($P<0.05$), TNB3 ($P<0.01$) and NBA3 ($P<0.01$) in Durocs only. The sows with *CC* genotype delivered significantly more TNB2 and TNB3 piglets than did those of the other two genotypes. What's more, the TNB1 and NBA3 numbers of *CC* genotype sows were significantly

Table 4. Statistical analysis of relation between genotypes at g.2612T>C in *ZAR1* gene and litter size in Duroc pigs

Litter size	Genotype	First litter	Second litter	Third and later litters
TNB	TT	7.48±0.37^{ab}	7.50±0.52^{b}	8.48±0.44^{B}
	CC	8.57±0.66^{a}	10.17±0.90^{a}	10.80±0.67^{A}
	CT	6.56±0.41^{b}	7.94±0.53^{b}	7.57±0.36^{B}
NBA	TT	7.43±0.36	7.33±0.50	8.43±0.44^{AB}
	CC	8.14±0.65	9.67±0.86	10.30±0.66^{A}
	CT	6.44±0.41	7.88±0.51	7.57±0.35^{B}

TNB – total number of piglets born; NBA – total number of piglets born alive.
$^{aA...}$Within columns means bearing different superscripts differ significantly at: small letters – $P<0.05$; capitals – $P<0.01$.

higher than that of *CT* ones (Tab. 4). However, no significant difference in litter size was observed between different genotypes in Landrace and Large White sows (data not shown).

The results of association analysis between genotypes at g.3838_3839insTGCAG and litter size in Duroc pigs are shown in Table 5. For the first litter, both TNB1 and NBA1 of *NN* genotype sows were significantly higher ($P<0.01$) than that of *MM* genotype, and a similar tendency was detected in Landrace and Large White pigs, though it was not significant (data not shown). For the third and later litters, there were significantly more piglets (TNB and NBA, $P<0.05$) for the sows of *NN* than of *MN*. genotype. Furthermore, a similar trend occurred in Small Meishan, Qingping and Landrace pigs, though it was not significant (data not shown).

The improvement of reproduction (litter size) in pigs is limited only through the traditional breeding methods due to its very low heritability (h^2=0.01). However, current technologies enable to improve the efficiency of traditional selection methods by applying genetic markers through marker-assisted selection (MAS), which could be used to improve the reproductive performances of pigs. Some genes or genetic markers were reported to be associated with litter size in pigs, such as *estrogen receptor (ER)* gene [Rothschild *et al*. 1996], G261A and T302C SNPs [Yuan *et al*. 2007a] and a 18 bp deletion/insertion in intron 2 in *zona pellucida 3* (*ZP3*) gene [Yuan *et al*. 2007b], which had a relation with the sex-related hormones or their receptors, and thus affected ovulation and uterus capacity.

Tautz [1988] found that maternal genes were essential for the early embryo development. It was reported that maternal bovine *ZAR1* transcripts persisted during oocyte *in vitro* maturation and fertilization and in preimplantation embryo until the five- to eight-cell or morula stage [Pennetier *et al*. 2004]. Furthermore, an expression of a 126 bp fragment showing the similarity with *ZAR1* gene has been reported to be expressed throughout pre-implantation bovine embryos development as well as

Table 5. Statistical analysis of relation between genotypes at g.3838_3839insTGCAG and litter size in Duroc pigs

Litter size	Genotype	First litter	Second litter	Third and later litters
TNB	MM	5.40±0.73^{B}	6.85±0.68	7.90±0.53^{ab}
	NN	9.43±1.07^{A}	9.33±141	10.67±1.40
	MN	7.95±0.61^{AB}	7.53±0.63	7.32±0.44^{b}
NBA	MM	5.40±0.73^{B}	6.85±0.68	7.90±0.53^{ab}
	NN	9.43±1.06^{A}	9.33±1.41	10.67±1.40^{a}
	MN	7.91±0.60^{AB}	7.53±0.63	7.32±0.44^{b}

TNB – total number of piglets born; NBA – total number of piglets born alive.
$^{aA...}$Within columns means bearing different superscripts differ significantly at: small letters – $P<0.05$; capitals – $P<0.01$.

in ovary [Brevini *et al*. 2004]. In pigs, ZAR1 played a central role in early embryo development highlighted by expression pattern of full-length transcript in oocytes and early embryos [Uzbekova *et al*. 2006]. Those authors indicated the essential role of *ZAR1* gene in the oocyte-to-embryo transformation.

In the present study, two mutations were found. in porcine *ZAR1* gene. One is g.2612T>C transition in exon 3 and the other is g.3838_3839insTGCAG in intron 3. These results show that a variable genetic background exists between local Chinese land European breeds. For instance, g.2612T>C SNP is present only in European breeds, and it was significantly associated ($P<0.05$) with litter size in Duroc pigs. The sows with *CC* genotype had more NBA3, TNB1, TNB2 and TNB3 piglets than those of TT or/and TC genotypes. Three genotypes at the g.3838_3839insTGCAG mutation were found in two local Chinese and three European breeds, and this mutation had a significant ($P<0.05$) effect on litter size only in Duroc pigs. The litter size (TNB1, TNB3, NBA1 and NBA3) of sows with *NN* genotype was significantly higher than that of *MM* or/and *MN*. Our study indicated that *ZAR1* gene might be a potential important candidate gene related to litter size, and the two polymorphisms found in *ZAR1* gene were associated with the litter size in pigs. They may influence the porcine oocyte functions, fertilization and early embryonic development possibly *via* their effects on regulations of *ZAR1* gene transcriptions and expressions, though they do not cause any changes in amino acid sequence of ZAR1 protein. To better understanding of mechanisms including *ZAR1* gene role in early stages of embryo development, a further study on larger sample size and functional studies are required.

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