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Association of non-synonymous SNPs of *OPN* gene with litter size traits in pigs

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Abstract. Osteopontin (*OPN*) gene is a secreted phosphoprotein which appears to play a key function in the conceptus implantation, placentation and maintenance of pregnancy in pigs. The objectives of this study were to verify the non-synonymous single nucleotide polymorphisms (SNPs) and their association with litter size traits in commercial Thai Large White pigs. A total of 320 Thai Large White sows were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Three SNPs at c.425G>A, c.573T>C and c.881C>T revealed amino acid exchange rates of p.110Ala>Thr, p.159Val>Ala and p.262Pro>Ser, respectively, and were then segregated. These three SNPs were significantly associated with total number born (TNB) and number born alive (NBA) traits. No polymorphisms of the two SNP markers (c.278A>G and c.452T>G) were observed in this study. Moreover, the SNPs at c.425G>A and c.573T>C were found to be in strong linkage disequilibrium. The association of *OPN* with litter size emphasizes the importance of porcine *OPN* as a candidate gene for reproductive traits in pig breeding.

1 Introduction

Litter size traits are the most important traits for reproduction to increase economic yields with regard to the breeding of pigs. The improvement of these traits through conventional methods revealed a low rate of efficiency due to low levels of heritability (Okere and Nelson, 2002; Grandinson et al., 2003). In fact, fertility traits are affected by season, parity and breed as well as housing and feeding conditions (Knecht et al., 2015). The usefulness of molecular genetics may generate a marker to improve the reproductive performance traits in pigs. Currently, both genomic and transcriptomic (microarray and RNA sequencing) approaches have been used to analyze quantitative trait loci (QTL) and to assess candidate genes for reproductive traits in pigs. Several genes have been discovered. One of these genes is osteopontin (*OPN*) which can be differentially expressed in various reproductive tissues, e.g., ovarian follicles, ovaries, myometrium and endometrium (Caetano et al., 2004; Fernandez-Roddriguez et al., 2011; Sun et al., 2011; Wang et al., 2013; Samborski et al., 2013).

The *OPN* or secreted phosphoprotein 1 (*SPP1*) gene is an extracellular matrix (ECM) protein and intergrin-binding ligand, as well as a highly phosphorylated acidic glycoprotein that stimulates cell–cell adhesion and increases cell–ECM communication, and promotes cell migration, cell signaling and remodeling in the uterus and placenta (Garlow et al., 2002; Johnson et al., 2003; White et al., 2005). The *OPN* gene is located on the long arm of the *Sus scrofa* chromosome 8 (SSC8q) and is composed of seven exons and six introns, which are encoded with a protein of 303 amino acids (Ensembl database: http://asia.ensembl.org/Sus_scrofa/Info/Index). The expression levels of *OPN* mRNA and the protein are increased in the uterus of pigs during pregnancy (Garlow et al., 2002; White et al., 2005). Some polymorphisms of the porcine *OPN* gene have been identified within a 5′-

Table 1. Primer sequence, PCR product size and restriction enzymes of SNPs markers of the porcine *OPN* gene.

SNP ID	SNP position	Location	Primer sequence	PCR product size (bp)	Restriction enzyme
rs341073067	c.278A>G	Exon 5	F: 5′-GCCATCCACCAATGAGGCTA-3′ R: 5′-ATGCCGGACCTTGGATTCAG-3′	189	*Taa*I
rs81509049	c.425G>A	Exon 6	F*: 5′-TCCGAGGAAGCTGATCGCG-3′	188	*Hin6*I
rs81214160	c.452T>G		R*: 5′-GATTTTGACCTCAGTCCGT-3′		*Hinf*I
rs81509053	c.573T>C				*Pfl23*II
rs81214162	c.881C>T	Exon 7	F : 5′-AGGACAGTCAGGAGACGAG-3′ R: 5′-TTCTTCGCTCTTAGAGTCTG-3′	200	*Tas*I

* Mismatch bases are underlined

flanking region and the promoter including exon 6 and intron 6 (Muráni et al., 2009; Goluch et al., 2009; Korwin-Kossakowska et al., 2013). Additionally, a highly polymorphic site of porcine *OPN* gene (ENSSSCG00000009216) has been reported in the Ensembl database (http://asia.ensembl.org/index.html). A total of 353 single nucleotide polymorphisms (SNPs) of the porcine *OPN* gene were identified (consisting of 5 missense, 7 synonymous, 5 3′-UTR, 132 intron, 100 upstream and 104 downstream gene variants; online date 10.04.2015). The short interspersed nuclear element (SINE) polymorphisms in the intron 6 and microsatellite markers of the porcine *OPN* gene have only been used to test for an association with the total number born (TNB), number born alive (NBA) and the number of piglets weaned (NW) as well as sperm quality traits in pig breeds (Short et al., 1997; Southwood et al., 1998; Knoll et al., 1999; Hamann et al., 2000; Putnova et al., 2001; Korwin-Kossakowska et al., 2002; Lin et al., 2006; Niu et al., 2008). Presently, there has still been no validation of the SNPs on the coding sequence of the porcine *OPN* gene (especial missense or non-synonymous mutation) as well as in terms of their association with the litter size traits in pigs. The objective of this study was to elucidate the effects of non-synonymous polymorphisms in the coding sequence of the porcine *OPN* gene on litter size traits in Thai commercial breed pigs.

2 Material and methods

2.1 Animals and DNA extraction

Blood samples were taken from a total of 320 sows of the Thai Large White pig breed. All animals were obtained from Betagro Hybrid International Company, Thailand. The reproductive performance traits of the sows were recorded in terms of litter size traits consisting of total number born (TNB) and number born alive (NBA). Genomic DNAs were extracted using the Chelex method (Walsh et al., 1991) and kept at 4 °C until analyzed.

2.2 Single nucleotide polymorphisms genotyping

The single nucleotide polymorphisms of the porcine *OPN* gene were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The specific primers were designed based on the porcine *OPN* nucleotide sequences (GenBank accession number: NM_214023) as shown in Table 1. Additionally, the mismatched primers were designed to generate a recognition site of the restriction enzyme for genotyping (Table 1). These designed primers covered five non-synonymous SNPs of the porcine *OPN* gene (c.278, c.425, c.452, c.573 and c.881; http://asia.ensembl.org/index.html).

The PCR was performed in a final volume of 20 µL containing 50 ng of genomic DNA sample, 1×NH_4SO_4 buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer and 0.2 U *Taq* DNA polymerase (Fermentas). The PCR conditions were 94 °C for 3 min denaturing, followed by 40 cycles of 94 °C for 30 s denaturing, 55 °C for 30 s annealing, 72 °C for 30 s extension and then 5 min at 72 °C to complete the extension. The PCR products were digested with 2.5 U of the restriction enzyme (Fermentas) for each primer and incubated for 2 h (Table 1). The digested fragments were separated on a 8 % polyacrylamide gel electrophoresis in 1×TBE buffer and stained with ethidium bromide.

2.3 Statistical analysis

The genotype and allele frequencies were calculated for all SNPs. The haplotype and linkage disequilibrium (LD) of each SNPs in the porcine *OPN* gene were analyzed using Haploview software version 4.2 with the solid spine of the LD method (Barrett et al., 2005). Association analysis of the *OPN* gene and the litter size traits was performed using a general linear model (GLM) of the SAS software package version 9.0 (SAS Institute Inc., Cary, NC, USA), including the fixed effects of season of the year, parity, genotype and residual error. The following model was used:

$$Y_{ijkl} = \mu + S_i + P_j + G_k + e_{ijkl}, \quad (1)$$

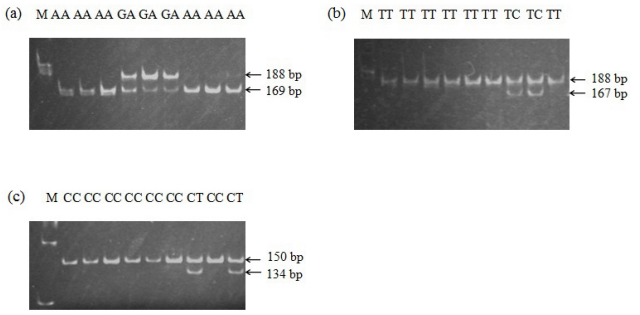

Figure 1. Genotyping SNPs of the porcine *OPN* gene **(a)** at c.425G>A locus with *Hin6*I **(b)** at c.573T>C locus with *Pfl23*II and **(c)** at c.881C>T locus with *Tas*I. The molecular marker of 100 bp DNA ladder (M) and the *OPN* genotypes are indicated at the top of each lane.

where Y_{ijkl} is representative of the observed values of the phenotype traits, μ represents the average normalized record of the populations, S_i represents the fixed effect of season of the year ($i = 1$–7), P_j represents the fixed effect of the parity ($j = 1$ and ≥ 2), G_k is the fixed effect of genotype ($k = 1$–3), and e_{ijkl} is representative of the residual error. The additive effect (a) was estimated by the comparison of the means of the trait value for a homozygote: $a = \frac{1}{2}$ (BB–AA) and the dominance effect (d) for the alleles A and B was calculated from the means of the following three genotypes: $d = \text{AB} - \frac{1}{2}$ (AA+BB). The estimated effects were evaluated using a t test on the significant deviation from zero (Lin et al., 2006).

3 Results

3.1 Verification of porcine *OPN* polymorphisms

In order to verify the polymorphisms of the porcine *OPN* gene, five non-synonymous SNPs were selected to be tested in the Thai commercial pig population. Three polymorphic sites of the porcine *OPN* gene were segregated among Thai Large White pigs, consisting of c.425G>A, c.573T>C and c.881C>T (Fig. 1). These polymorphisms revealed a non-synonymous mutation leading to a non-conservative amino acid exchange at the position p.110Ala>Thr, p.159Val>Ala and p.262Pro>Ser, respectively. No polymorphisms of the two SNP markers (c.278A>G and c.452T>G) were observed in this study.

3.2 Genotype and allele frequencies of porcine *OPN* gene

The genotype and allele frequencies of the porcine *OPN* gene at each polymorphic site are shown in Table 2. Three SNPs were found to be segregated among Thai Large White sows. At the c.425 and c.881 loci, three genotypes were found to be present, whereas at the c.573 locus, two genotypes were present in this pig population. Alleles of c.425G, c.573T and c.881C were the major alleles in this pig breed. Moreover, at the c.278 and c.452 loci, these two SNPs were fixed as c.278A and c.452T among Thai Large White sows.

Table 2. Genotype and allele frequencies of the porcine *OPN* gene in Thai Large White sows.

Locus	Genotype frequency			Allele frequency	
	AA	AB	BB	A*	B
c.278A>G	1.00	0.00	0.00	1.00	0.00
c.425G>A	0.19	0.39	0.42	0.38	0.62
c.452T>G	0.00	0.00	1.00	0.00	1.00
c.573T>C	0.87	0.13	0.00	0.93	0.07
c.881C>T	0.69	0.26	0.05	0.82	0.18

* Allele A represents wild type alleles of c.278A, c.425G, c.452T, c.573T and c.881C for each locus and allele B represents mutate alleles of c.278G, c.425A, c.452G, c.573C and c.881T for each locus.

3.3 Haplotype analysis

Haplotype block and graphical representation of LD structure were generated. A haplotype block was found in the porcine *OPN* gene (Fig. 2). There was a strong linkage disequilibrium (LD) found between the SNPs at position c.425 and c.573 ($\text{D}' = 0.85$). On the other hand, the SNPs c.425 and c.881 or the c.573 and c.881 were found to be in the moderate range of LD ($\text{D}' = 0.66$, $\text{D}' = 0.76$, respectively) among Thai Large White pigs.

3.4 Association of porcine *OPN* polymorphisms with litter size traits

The results of the association analysis between three SNPs (c.425, c.573 and c.881) and litter size traits are shown in Table 3. No significant association of SNPs with litter size traits were observed in the first parity of pigs. In later parities, these three SNPs were significantly associated ($P < 0.05$) with the TNB and NBA traits of sows. At the c.425 locus, the sows with the AA and GA genotypes had significantly higher TNB and NBA values than those of the sows with the GG genotype. At the c.573 locus, the sows with the TT genotype revealed significantly higher TNB and NBA values than those of the sows with the TC genotype. At the c.881 locus, the sows with the CC and CT genotypes had significantly higher TNB and NBA values than those of the sows with the TT genotype. The significant additive and dominance effects for TNB and NBA were detected in later parities at the c.425 locus. Moreover, the significant additive effect for NBA was observed in later parities at the c.881 locus. Effects of genotype combinations of c.425, c.573 and c.881 on litter size traits are shown in Table 4. No association of genotype combinations with litter size traits for the first parity were seen. In later parities, genotype combinations of these SNPs were sig-

Table 3. Association of the porcine *OPN* gene with litter size traits.

SNPs	Parity	Traits	Genotypes (mean ± SE)			Additive	Dominance
			GG	GA	AA		
c.425	First parity	TNB	9.77 ± 0.82	10.79 ± 0.52	10.32 ± 0.58	−0.27± 0.49	0.74 ± 0.25
		NBA	8.67 ± 0.89	9.40 ± 0.56	9.67 ± 0.63	−0.50± 0.35	0.23 ± 0.11
	Later parities	TNB	10.90 ± 0.39^{a}	12.49 ± 0.19^{b}	12.29 ± 0.21^{b}	-0.69 ± 0.21^{d}	0.89 ± 0.41^{c}
		NBA	9.72 ± 0.38^{a}	11.46 ± 0.19^{b}	11.19 ± 0.20^{b}	-0.73 ± 0.21^{c}	1.00 ± 0.29^{d}
			TT	TC	CC		
c.573	First parity	TNB	10.51 ± 0.38	10.55 ± 0.94	0	–	–
		NBA	9.36 ± 0.48	10.00 ± 1.01	0	–	–
	Later parities	TNB	12.43 ± 0.14^{a}	10.26 ± 0.44^{b}	0	–	–
		NBA	11.33 ± 0.14^{a}	9.52 ± 0.44^{b}	0	–	–
			CC	CT	TT		
c.881	First parity	TNB	10.50 ± .45	10.23 ± 0.58	10.99 ± 1.89	−0.24± 0.37	−0.51± 0.12
		NBA	9.73 ± 0.47	8.58 ± 0.61	11.34 ± 0.34	−0.80± 0.23	0.95 ± 0.48
	Later parities	TNB	12.48 ± 0.16^{a}	11.72 ± 0.24^{a}	10.86 ± 0.95^{b}	0.81 ± 0.44	0.04 ± 0.50
		NBA	11.45 ± 0.15^{a}	10.57 ± 0.23^{a}	8.91 ± 0.92^{b}	1.27 ± 0.47^{c}	0.38 ± 0.52

Mean ± SE represents least square mean ± standard error. [a, b] Values in each row are significantly different ($P < 0.05$). [c, d] Statistically significant differences of the additive and dominance effects; [c] $P < 0.01$; [d] $P < 0.001$.

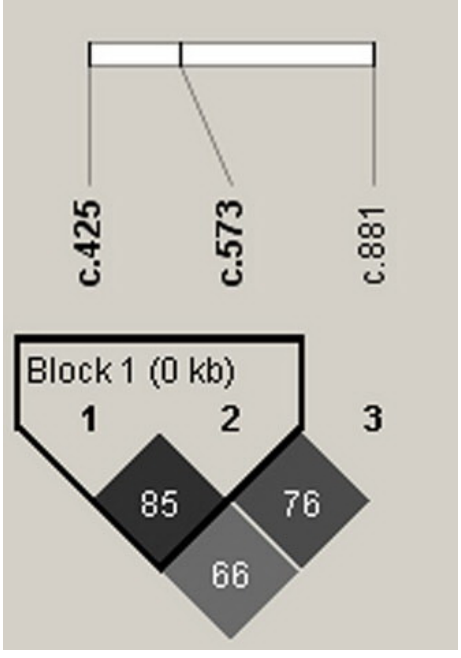

Figure 2. Linkage disequilibrium (LD) plot of three SNPs (c.425, c.573 and c.881) of the porcine *OPN* gene. The values in squares represent the pairwise D′ values and the haplotype block is indicated by a stronger LD.

nificantly associated with TNB and NBA traits in Thai Large White sows. The number of favorable alleles was related with the TNB and NBA traits.

4 Discussion

The function of the *OPN* gene is involved in uterine endometrial glandular epithelium, adhesion and communication between the conceptus trophectoderm with uterine endometrial luminal epithelium during early implantation period, influence fetal/placental development, growth and mediate communication between placental and uterine tissues that support pregnancy (Garlow et al., 2002; Johnson et al., 2003). Furthermore, the *OPN* gene corresponds to the location with the QTL regions for reproductive traits, such as ovulation rate (Rathje et al., 1997), uterine capacity (Rohrer et al., 1999), age of puberty (Cassady et al., 2001), litter size and embryo-survival rate (King et al., 2003), on SSC8 in pigs. Moreover, the function genomic analysis showed that the differential expression levels of the *OPN* gene were found in the ovarian follicles, ovaries, myometrium and endometrium (Caetano et al., 2004; Fernandez-Roddriguez et al., 2011; Sun et al., 2011; Wang et al., 2013; Samborski et al., 2013). Therefore the *OPN* gene can be regarded as a candidate gene for the determination of reproductive traits in pigs.

The polymorphisms of the porcine *OPN* gene have been identified (Knoll et al., 1999; Zhang et al., 1992). The SINE polymorphisms at intron 6 of the porcine *OPN* gene were found to be associated with litter size as well as body weight at birth and weaning traits (Korwin-Kossakowska et al., 2002; Niu et al., 2008; Zhang et al., 2010). These SINE polymorphisms were found to be associated with the uterine weight in Polish Landrace pigs (Kapelanski et al., 2013). Moreover, two synonymous SNP (c.559A>C and c.574C>G) of the porcine *OPN* gene were associated with their expres-

Table 4. Association of genotype combinations of SNP c.425, c.573 and c.881 with litter size traits.

Genotype combinations	Parity	Traits	Number of favorable alleles (mean ± SE)				
			0	1	2	3	4
c.425-c.573	First parity	TNB	–	10.19 ± 1.23	10.33 ± 0.96	10.56 ± 0.56	10.48 ± 0.62
		NBA	–	10.21 ± 1.13	8.37 ± 1.02	9.23 ± 0.60	9.85 ± 0.66
	Later parities	TNB	–	10.21 ± 0.57^{a}	11.10 ± 0.44^{a}	12.56 ± 0.19^{b}	12.33 ± 0.21^{b}
		NBA	–	9.50 ± 0.56^{a}	9.77 ± 0.44^{a}	11.50 ± 0.19^{b}	11.23 ± 0.21^{b}
c.425-c.881	First parity	TNB	10.98 ± 1.87	9.49 ± 0.93	10.76 ± 0.19	10.74 ± 0.57	10.22 ± 0.67
		NBA	11.46 ± 1.99	7.91 ± 0.99	9.25 ± 0.94	9.34 ± 0.60	9.95 ± 0.72
	Later parities	TNB	10.81 ± 0.95^{ab}	10.90 ± 0.44^{a}	12.14 ± 0.37^{b}	12.47 ± 0.20^{b}	12.39 ± 0.23^{b}
		NBA	8.87 ± 0.93^{a}	9.77 ± 0.43^{a}	11.03 ± 0.36^{b}	11.46 ± 0.19^{b}	11.28 ± 0.22^{b}
c.573-c.881	First parity	TNB	–	11.40 ± 2.16	9.96 ± 1.09	10.65 ± 0.66	10.51 ± 0.46
		NBA	–	11.79 ± 2.30	9.22 ± 1.16	8.72 ± 0.70	9.74 ± 0.49
	Later parities	TNB	–	11.29 ± 0.23^{abc}	9.91 ± 0.54^{a}	11.90 ± 0.26^{b}	12.58 ± 0.15^{c}
		NBA	–	10.34 ± 0.21^{abc}	8.76 ± 0.53^{a}	10.75 ± 0.26^{b}	11.53 ± 0.15^{c}

Mean ± SE represents least square mean ± standard error. Values in each row with different superscript letters are significantly different ($P < 0.05$). Number of favorable alleles is accumulated alleles of combination genotypes for c.425A, c.573T and c.881C.

sion levels in uterine tissues (Korwin-Kossakowska et al., 2013). Additionally, the microsatellite markers of this gene were associated with the TNB and NBA traits in sows (Short et al., 1997; Southwood et al., 1998) as well as boar fertility traits (Lin et al., 2006).

Several polymorphisms in the 5′-flanking region and promoter of the porcine *OPN* gene have also been analyzed (Muráni et al., 2009; Goluch et al., 2009; Korwin-Kossakowska et al., 2013). The g.3836A>G locus regulated the porcine *OPN* expression and affected the CCAAT/enhancer binding protein beta (C/EBPβ) responsive transcriptional enhancer (Muráni et al., 2009). Moreover, two SNPs at positions g.1999A>G (−617A>G) and g.2011A>G (−606 A>G) loci of the porcine *OPN* promoter region were associated with mRNA expression levels in the uterus (Korwin-Kossakowska et al., 2013).

In this study, three of five non-synonymous SNPs (c.425G>A, c.573T>C and c.881C>T) of the porcine *OPN* gene were found to be segregated in Thai Large White sows. The c.425 and c.573 SNPs revealed strong linkage disequilibrium. These three SNPs (c.425G>A, c.573T>C and c.881C>T) of the porcine *OPN* gene showed a significant association with the TNB and NBA traits. The positive effects of the favorable c.425A, c.573T and c.881C alleles on TNB and NBA were found in later parities. The increased number of favorable alleles was associated with the TNB and NBA traits in sows. Moreover, the high frequency of the allele c.425A, c.573T and c.881C were observed in this population of sows. These results indicate a relatively high frequency of these alleles along with the high selection pressure for litter size traits in this commercial pig breed. Moreover, two SNPs (g.1999A>G and g.3836A>G) of the 5′-flanking region and the promoter were also detected in this study revealing a lower polymorphisms in Thai Large White pigs (data not shown).

In this study, the association of non-synonymous SNPs at the c.573T>C locus of the porcine *OPN* gene with litter size traits is of a significant level of interest due to the changing amino acid of p.159Val>Ala located on the ^{154}RGDSVVYGLR^{163} integrin binding sites, which is bound to integrin receptors on conceptus trophectoderm and uterine luminal epithelium. It is involved in conceptus elongation and implantation (Erikson et al., 2009). The c.573T>C polymorphism may be distrusted in the molecular binding mechanism of the intergrin receptors and their ligand of the conceptus and uterine to promote trophectoderm cell migration and the attachment to luminal epithelium. Our results indicate that the non-synonymous polymorphisms of the porcine *OPN* gene are significantly correlated with litter size traits. This SNP may directly play an important function in the early stage of the development of the term of pregnancy in pigs. Further studies should be conducted on the effects of these SNPs on protein function as well as molecular affinity for its target molecules.

In conclusion, we report on the non-synonymous SNPs in the porcine *OPN* gene and their associations with litter size traits. The SNPs at c.425G>A, c.573T>C and c.881C>T loci were found to be associated with the TNB and NBA traits in Thai Large White pigs. Moreover, the SNPs at c.425 and c.573 were shown to be in strong linkage disequilibrium. These findings emphasize the importance of the porcine *OPN* gene in the reproductive traits of pigs. Therefore, these SNPs of the porcine *OPN* gene may be a potential candidate gene for the purposes of increasing litter size traits in the breeding of pigs.

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