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Research article

Association of osteopontin gene with intramuscular fat content and fatty acid composition traits in pigs

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Abstract

Osteopontin (OPN) is a secreted phosphoprotein that is involved in the development of skeletal muscle and fat deposition. The objectives of this study were to identify the polymorphism of the *OPN* gene and to analyze the association of the *OPN* gene with intramuscular fat (IMF) content and fatty acid (FA) composition in pigs. *Longissimus thoracis* (LT) muscle samples taken from the 10-11th rib were collected from a total of 328 Duroc pigs. Genomic DNA samples were extracted from LT muscle tissues using the phenol-chloroform method. IMF content was measured using the ether extraction method and FA composition was measured by gas chromatography. The porcine *OPN* polymorphisms were identified by DNA sequencing and were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The association analysis of the *OPN* gene with IMF and FA composition traits was performed using a general linear model (GLM). Two polymorphic sites (*OPN* g.2442-2471indel and g.3836A>G) were found in the 5′-flanking region and intron 1 of the porcine *OPN* gene. The *OPN* g.2442-2471indel polymorphism was found to be significantly associated with IMF content and ω3 FA levels ($P<0.05$). Moreover, *OPN* g.3836A>G polymorphism was significantly associated with the linolenic acid levels in the muscles of pigs ($P<0.05$). The results of this study indicate that the *OPN* gene is important to IMF content, as well as linolenic and ω3 FA levels in pigs, and could be used as a candidate gene to improve fat deposition and fatty acid composition in the muscles of pigs.

Keywords: Fatty acid composition, Intramuscular fat, Osteopontin, Pig

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INTRODUCTION

Intramuscular fat (IMF) content is an economically important trait for the determination of meat quality and is strongly related to the eating quality of meat (Ros-Freixedes et al., 2014; Wood et al., 2004). Moreover, fatty acid (FA) composition is considered an indicator of lipid quality and is closely related to the nutritional value of pork (Sanchez et al., 2007; Wood et al., 2004). The IMF is defined as the total lipid associated with all cells present in a meat sample, excluding adipocytes from the intermuscular fat (Gao and Zhao, 2009) whereas, the FA composition of muscle is amount of fatty acid components in total lipid of IMF. High levels of IMF content and FA composition especially, palmitoleic, oleic, and monounsaturated fatty acid (MUFA) are positively associated with the flavor of pork (Cameron et al., 2000; Wood et al., 2004; Zhang et al., 2019). Currently, the genome-wide association study (GWAS) has been used to identify candidate genes for IMF and FA content in the muscle tissue of pigs (Ding et al., 2019; Zhang et al., 2016; Zhang et al., 2019). Moreover, the entire transcriptomic approach has been analyzed along with numerous genes that are related to IMF content and FA composition in various pig breeds (Ropka-Molik et al., 2015). One of these genes is porcine osteopontin (*OPN*), which is known to be differently expressed in muscle and adipose tissues (Ropka-Molik et al., 2015).

The *OPN* or secreted phosphoprotein 1 (*SPP1*) gene is a member of the cytokine family and is expressed in macrophage, skeletal muscle, and adipose tissues (Gómez-Ambrosi et al., 2007; O'Brien et al., 1994; O'Regan et al., 1999; Xu et al., 2005). It plays a major role in immune responses, apoptosis, and cell-cell signaling (Ropka-Molik et al., 2014). The porcine *OPN* gene has been mapped on *Sus scrofa* chromosome 8 (SSC8). The coding sequence of the porcine *OPN* gene is 1507 bp in length. It is composed of seven exons and six introns and was found to encode a peptide of 303 amino acids (ENSSSCT00000010091.5; Ensembl data base; https://asia.ensembl.org/). Previous studies have reported that the polymorphism of porcine *OPN* is associated with reproductive and litter size traits in pigs (Knoll et al., 1999; Korwin-Kossakowska et al., 2002; Kumchoo and Mekchay, 2015; Niu et al., 2008). However, it has been demonstrated that the *OPN* gene is up-regulated in the muscle tissues of lean pigs (Muráni et al., 2009; Ropka-Molik et al., 2014). Moreover, *OPN* is also expressed in backfat and intramuscular fat tissues in pigs (Sun et al., 2013; Wu et al., 2013). The polymorphism of the porcine *OPN* gene is associated with the loin eye area and backfat thickness (Han et al., 2012). Thus, the *OPN* gene may play an important role in adipogenesis in skeletal muscle tissues and backfat tissues (Han et al., 2012). However, available information on the association of the porcine *OPN* gene with IMF content and FA composition traits in pigs has been limited. In the present study, polymorphisms of the porcine *OPN* gene have been identified and their association with IMF and FA composition traits has also been assessed in commercial Duroc pigs.

MATERIALS and METHODS

Animals and phenotypic determination

A total of 328 pigs were obtained from a group of purebred Duroc pigs (158 barrows and 170 gilts) that were reared under the commercial conditions of the Betagro Hybrid International Company, Thailand. All the pigs were fed the same corn-soybean-based diet containing 3200 kcal/kg digestible energy and 16% crude protein. The pigs were slaughtered according to standard commercial procedures at a body weight of about 90 kg (153±1.98 days of age). Thus, ethical approval was not required for this investigation. *Longissimus thoracis* (LT) muscle tissues obtained from the 10-11th rib were taken to analyze IMF content and FA composition (Figure 1). The genomic DNA samples were isolated from LT muscle tissues using the standard phenol-chloroform protocol. The degree of concentration of the DNA sample was measured with a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). The LT muscle tissues (10 g) were freeze-dried and pulverized prior to IMF analysis. IMF content was determined using the ether extraction method (AOAC, 2000) and expressed as the percentage of IMF of the dry matter of muscle tissues. Fatty acid composition was measured with the method according to a previous study (Zappaterra et al., 2020) by using a gas chromatography-flame ionization detector (SCION 456-GC, Bruker Daltonics Inc., Fremont, USA) with an RT-2560 capillary column (100 m length, 0.25 mm internal diameter, 0.2µm film thickness, RESTEK, Bellefonte, PA, USA). Injector and detector temperatures were held at 250°C. The column oven temperature program was increased from 50 to 220°C at a rate of 10°C/min, held for 35 min, then increased from 200 to 230°C at a rate of 5°C/min and held at 230°C for 20 min. The sample volume of 1 µl was injected. A 37-component standard FAME mix (Food Industry Fame Mix, RESTEK, Bellefonte, PA, USA) was used for the identification of FAMEs. FA composition was expressed as the percentage of total fatty acids.

Figure 1 Pork loins from the *longissimus thoracis* muscle tissues with (A) high- and (B) low-intramuscular fat content.

Polymorphisms of porcine *OPN* gene and genotyping

To identify polymorphisms in the porcine *OPN* gene, specific primers were designed based on the available sequence information (GenBank accession number: M84121.1). Ten DNA samples of the 5-highest and 5-lowest IMF content levels (11.96±0.26% vs 6.28±0.19%) were selected and used to amplify the DNA fragments of the porcine *OPN* genes using each primer (Table 1). The amplicons of the porcine *OPN* gene were sequenced using the CEQ 8000 Genetic Analysis System (Beckman-Coulter, USA) in order to identify the SNP variants in this pig population. Two single nucleotide polymorphisms (SNPs) of the porcine *OPN* gene (g.2442-2471indel and g.3836A>G) were genotyped in 328 Duroc pigs. Polymerase chain reaction (PCR) amplification was carried out in a total volume of 20 µL consisting of 50 ng of genomic DNA sample, 1×$(NH_4)_2SO_4$ buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM each primer (Table 2), and 0.2 U *Taq* DNA polymerase (Fermentas, USA). The PCR conditions were as follows: 95°C for 3 min, followed by 32 cycles of denaturation at 95°C for 30 sec, annealing at 58-60°C for 30 sec, elongation at 72°C for 45 sec, and then 5 min at 72°C to complete the reaction. The PCR products of the porcine *OPN* g.3836A>G gene were digested with restriction enzymes (Table 2). The PCR products and digested products were electrophoresed on 6% polyacrylamide gels and stained with ethidium bromide for visualization.

Statistical analysis

The genotype and allele frequencies of the porcine *OPN* polymorphisms were estimated. Effects of the porcine *OPN* polymorphisms on IMF content and FA composition traits were analyzed with a general linear model as expressed below: $Y_{ijk} = \mu+S_i+G_j+e_{ijk}$ where Y_{ijk} represents the observations of the phenotype values, µ is representative of the overall mean for each trait, S_i represents the fixed effect of sexes, G_j represents the fixed effect of the *OPN* genotypes, and e_{ijk} represents the residual error. The effects were estimated by *t*-tests on significant deviations from zero ($P<0.05$).

Table 1 Primer sequences used for polymorphism identification in the porcine *OPN* gene.

Primers	Nucleotide sequence	Location (bp)	Tm (°C)	Product size (bp)
OPN-1	F: 5′-GCTACATCTGGAGTTCCCAT-3′	1237-1256	60	242
	R: 5′-AAGGGCTGCACATGCTTCA-3′	1460-1478		
OPN-2	F: 5′-TCTAATTCAGAGTTAATATAGTC-3′	2273-2295	58	383
	R: 5′-TGATGCGGCTGTCAGTGCT-3′	2637-2655		
OPN-3	F: 5′-TGCCTAACAGTAAGAGATGG-3′	3621-3640	58	323
	R: 5′-GAATTCTGTTTAAGATTCAGCT-3′	3922-3945		

Table 2 Primer sequences, PCR condition, and restriction enzyme used for genotyping of the porcine *OPN* gene.

OPN markers	Nucleotide sequence	Location (bp)	Tm (°C)	Product size (bp)	Restriction enzyme
g.2442-2471indel	F: 5′-CCAATCCTATTCACGAAAAAG-3′	2375-2395	58	142	-
	R: 5′-CAACCCACTTGCTCCCAC-3′	2499-2516			
g.3836A>G	F: 5′-GTAGTAGAGAGTATTTCTATAG-3′	3711-3732	58	149	*Tsp*509I
	R*: 5′-GGTAATGATTTTCCTGCAAAAAA-3′	3837-3859			

*Mismatched nucleotide is underlined to generate a recognition site of the restriction enzyme *Tsp*509I for genotyping.

RESULTS

Polymorphisms of porcine *OPN* gene

Two SNP loci of the porcine *OPN* gene were found to be segregated among this Duroc population. An indel of GT polymorphisms of the porcine *OPN* gene was found in the *OPN*-2 DNA fragments of the 5′-flanking region at position g.2442-2471indel. This fragment length polymorphism was detected with PCR and gel electrophoreses (Figure 2). Four motif alleles were presented as $(GT)_{14}$, $(GT)_{20}$, $(GT)_{35}$, and $(GT)_{46}$ with PCR product sizes of 142, 154, 184, and 206-bp, respectively. A polymorphic site of g.3836A>G was found in the *OPN*-3 DNA fragments of intron 1 of the porcine *OPN* gene. This was detected with the restriction enzyme *Tsp*509I (Figure 3). Two specific alleles exhibited a 149-bp fragment for allele A and two fragments of 125 and 24-bp for allele G.

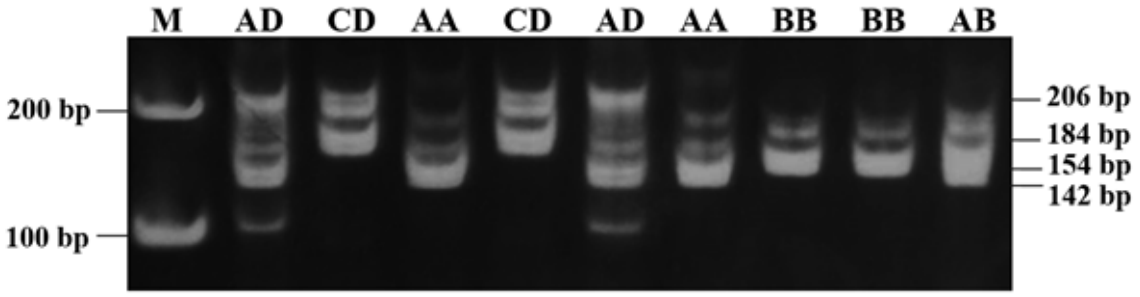

Figure 2 Genotyping SNP of porcine *OPN* g.2442-2471indel locus. The molecular marker of 100 bp DNA ladder (M) and the genotypes of porcine *OPN* g.2442-2471indel SNP are indicated at the top of each line. A 142-bp fragment for allele A, a 154-bp fragment for allele B, a 184-bp fragment for allele C, and a 206-bp fragment for allele D.

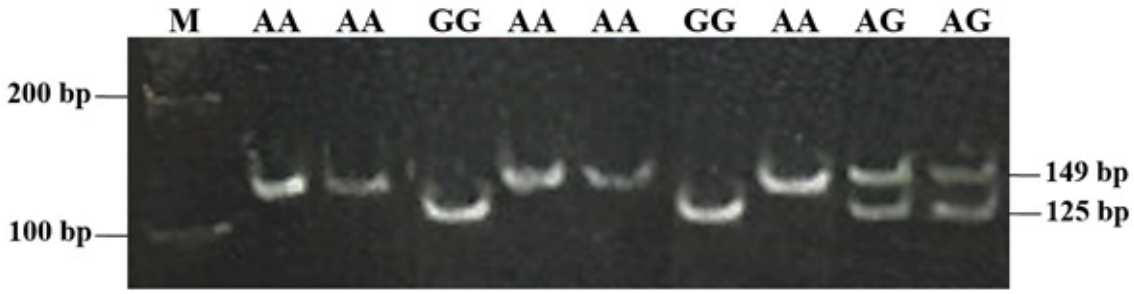

Figure 3 Genotyping SNP of porcine *OPN* g.3836A>G locus with *Tsp*509I. The molecular marker of 100 bp DNA ladder (M) and the genotypes of porcine *OPN* g.3836A>G SNP are indicated at the top of each line. A 149-bp fragment for allele A and two fragments of 125 and 24-bp for allele G. Notably, the 24-bp fragment is not shown in the gel.

Genotype frequencies

The genotype frequencies of the porcine *OPN* gene are shown in Table 3. At the *OPN* g.2442-2471indel locus, five genotype patterns were presented as 142/142, 154/154, 142/154, 142/206, and 184/206-bp. The *OPN* g.2442-2471indel with 142/154 genotype was found to be more frequent in this pig population. At the *OPN* g.3836A>G locus, three genotypes were observed. The *OPN* g.3836A>G polymorphism with GG genotype was found to be more frequent in these Duroc pigs.

Association of porcine *OPN* gene with IMF content and fatty acid composition

The effects of porcine *OPN* g.2442-2471indel and g.3836A>G polymorphisms on IMF content and FA composition traits were assessed in Duroc pigs. No significant effect of the sex factor on IMF content and FA composition was found in this Duroc population. Association of the porcine *OPN* g.2442-2471indel and g.3836A>G polymorphisms with IMF content and FA composition is shown in Tables 4 and 5. The porcine *OPN* g.2442-2471indel was significantly associated with IMF content and ω3 FA levels ($P<0.05$). The pigs with the 142/206 genotype had higher IMF content than the pigs with the 154/154 and 184/206 genotypes. Furthermore, the pigs with the 154/154, 142/154, and 184/206 genotypes had higher ω3 FA levels than the pigs with the 142/206 genotype. There was no significant association of the porcine *OPN* g.3836A>G polymorphism with IMF content in these Duroc pigs. However, the porcine *OPN* g.3836A>G polymorphism was significantly associated with linolenic acid levels. Moreover, the pigs with AG revealed higher linolenic acid levels than the pigs with the AA and GG genotypes ($P<0.05$).

Table 3 Genotype frequencies of the porcine *OPN* g.2442-2471indel and the porcine *OPN* g.3836A>G in pigs.

Markers	Genotype frequencies				
OPN g.2442-2471indel	142/142	154/154	142/154	142/206	184/206
	0.09	0.33	0.41	0.07	0.10
OPN g.3836A>G	AA	AG	GG		
	0.30	0.31	0.39		

Table 4 Association of the porcine *OPN* g.2442-2471indel with IMF content and fatty acid composition traits in pigs.

Traits[1] (%)	Genotypes (least-squares mean± SE)					P-value
	142/142	154/154	142/154	142/206	184/206	
IMF	9.25±1.00^{ab}	7.77±0.51^{b}	8.62±0.80^{ab}	11.00±1.33^{a}	6.49±0.55^{c}	0.0198
Lauric (C12:0)	0.14±0.01	0.09±0.01	0.11±0.01	0.13±0.01	0.11±0.01	0.0560
Myristic (C14:0)	1.56±0.14	1.37±0.10	1.41±0.11	1.60±0.16	1.47±0.14	0.5808
Palmitic (C16:0)	17.75±1.05	14.10±0.64	15.76±0.93	16.07±1.05	15.57±1.13	0.1584
Stearic (C18:0)	12.69±0.89	13.09±0.55	11.05±0.79	12.50±0.89	11.42±0.96	0.3453
Arachidic (C20:0)	0.32±0.07	0.27±0.05	0.42±0.07	0.21±0.07	0.23±0.08	0.2693
SFA	32.54±1.23	28.74±0.75	28.67±1.32	30.39±1.23	28.68±1.08	0.1862
Palmitoleic (C16:1n-7)	5.66±0.49	3.95±0.30	4.50±0.43	5.37±0.49	4.67±0.53	0.1005
Oleic (C18:1n-9)	42.99±2.03	39.74±1.25	40.04±1.79	40.07±2.03	39.23±2.19	0.7894
Eicosenoic (C20:1n-9)	1.81±0.16	2.12±0.16	1.87±0.23	1.56±0.26	1.51±0.28	0.4174
MUFA	50.47±2.40	45.81±1.47	46.41±2.11	47.00±2.40	45.42±2.59	0.6566
Linoleic (C18:2n-6)	11.55±2.58	19.30±1.59	20.06±2.28	16.60±2.58	20.83±2.79	0.1559
Linolenic (C18:3n-6)	0.02±0.06	0.04±0.03	0.03±0.05	0.03±0.06	0.20±0.06	0.2256
Eicosadienoic (C20:2n-6)	1.24±0.19	1.52±0.11	1.42±0.16	1.25±0.19	1.14±0.20	0.2435
Homolinolenic (C20:3n-3)	0.13±0.07	0.21±0.04	0.20±0.06	0.12±0.07	0.16±0.07	0.6467
PU FA	12.95±2.52	21.08±1.55	21.71±2.23	18.00±2.52	22.34±2.72	0.1225
ω3 FA	0.60±0.07^{ab}	0.72±0.04^{a}	0.71±0.06^{a}	0.53±0.07^{b}	0.74±0.07^{a}	0.0370
ω6 FA	11.81±2.56	19.54±1.58	20.39±2.26	16.83±2.56	21.26±2.76	0.1421
ω9 FA	43.00±2.03	39.74±1.25	39.23±2.19	40.07±2.03	40.04±1.79	0.7894

[1]IMF: intramuscular fat content, SFA: saturated fatty acids (C14:0+C16:0+C18:0+C20:0), MUFA: monounsaturated fatty acids (C16:1n-7+C18:1n-9+C20:1n-9), PUFA: polyunsaturated fatty acids (C18:2n-6+C18:3n-6+C20:2n-6+C20:3n-6+C20:4n-6), ω3 fatty acids (C18:3n-3+C20:5n-3+C22:6n-3), ω6 fatty acids (C18:2n-6+C18:3n-6+C20:3n-6) and ω9 fatty acids (C18:1n-9). IMF was expressed as the percentage (%) of IMF content of the dry matter of muscle tissues. FA composition was expressed as the percentage (%) of total fatty acids. $^{a, b, c}$ Values in each row with different superscript letters are significantly different ($P<0.05$).

Table 5 Association of the porcine *OPN* g.3836A>G with IMF content and fatty acid composition traits in pigs.

Traits[1] (%)	Genotypes (least-squares mean± SE)			P-value
	AA	AG	GG	
IMF	7.77±0.51	7.21±0.62	8.61±0.60	0.3875
Lauric (C12:0)	0.10±0.01	0.11±0.01	0.11±0.01	0.6675
Myristic (C14:0)	1.43±0.09	1.36±0.11	1.43±0.08	0.3395
Palmitic (C16:0)	14.83±0.80	15.29±1.00	15.67±0.79	0.8937
Stearic (C18:0)	12.04±0.65	11.92±0.80	12.82±0.64	0.8159
Arachidic (C20:0)	0.33±0.05	0.24±0.07	0.30±0.05	0.6301
SFA	28.55±0.87	28.92±1.08	30.27±0.86	0.5698
Palmitoleic (C16:1n-7)	4.47±0.40	4.79±0.50	4.44±0.40	0.9532
Oleic (C18:1n-9)	40.88±1.36	40.22±1.69	39.85±1.35	0.9563
Eicosenoic (C20:1n-9)	1.98±0.17	1.68±0.21	1.98±0.17	0.6321
MUFA	47.34±1.66	46.68±2.07	46.27±1.64	0.9740
Linoleic (C18:2n-6)	18.67±2.03	18.78±2.52	16.82±2.01	0.8666
Linolenic (C18:3n-6)	0.03±0.03^{b}	0.17±0.04^{a}	0.01±0.03^{b}	0.0352
Eicosadienoic (C20:2n-6)	1.34±0.16	1.36±0.20	1.69±0.16	0.0815
Homolinolenic (C20:3n-3)	0.17±0.04	0.22±0.06	0.16±0.04	0.4937
PUFA	20.22±2.03	20.52±2.53	18.68±2.01	0.9236
ω3 FA	0.65±0.05	0.70±0.06	0.70±0.05	0.0650
ω6 FA	18.95±2.03	19.15±2.52	17.06±2.00	0.8502
ω9 FA	40.88±1.36	45.22±1.69	39.85±1.35	0.9563

[1]IMF: intramuscular fat content, SFA: saturated fatty acids (C14:0+C16:0+C18:0+C20:0), MUFA: monounsaturated fatty acids (C16:1n-7+C18:1n-9+C20:1n-9), PUFA: polyunsaturated fatty acids (C18:2n-6+C18:3n-6+C20:2n-6+C20:3n-6+C20:4n-6), ω3 fatty acids (C18:3n-3+C20:5n-3+C22:6n-3), ω6 fatty acids (C18:2n-6+C18:3n-6+C20:3n-6) and ω9 fatty acids (C18:1n-9). IMF was expressed as the percentage (%) of IMF content of the dry matter of muscle tissues. FA composition was expressed as the percentage (%) of total fatty acids. $^{a, b}$ Values in each row with different superscript letters are significantly different ($P<0.05$).

DISCUSSION

Duroc pigs are the most popular breed in the pig production due to its good growth and show superior IMF deposition. In this present study, we identified polymorphisms in the porcine *OPN* gene and assessed their effects on IMF content and FA composition in the LT muscle tissues of Duroc pigs. Two polymorphic sites of the porcine *OPN* g.2442-2471indel and g.3836A>G loci were segregated in these Duroc pigs. These two polymorphisms were located in a non-coding sequence (5′-flanking region and intron 1) and this corresponded with the uncovered SNPs of the previous study (Muráni et al., 2009). The porcine *OPN* g.2442-2471indel with the 142/154 and 154/154-bp genotypes was more frequent in this Duroc population. In contrast, the porcine *OPN* g.3836A>G locus revealed a similar genotype distribution among three genotypes (AA, AG, and GG) in this pig population. These results indicate that the porcine *OPN* g.2442-2471indel locus might be related to selective pressures on some desirable production traits. On the other hand, the porcine *OPN* g.3836A>G was under homeostasis accompanied by the effect of artificial selection. Moreover, the SNP variants at g.1804C>T, g.1999A>G, and g.2011A>G loci in the 5′-flanking region of the porcine *OPN* gene have been identified (Korwin-Kossakowska et al., 2013; Muráni et al., 2009). Currently, a

total of 1147 SNPs of the porcine *OPN* gene have been characterized (Ensembl database, Sscrofa 11.1, https://asia.ensembl.org/Sus_scrofa/Info/ Index, online access 16.09.2020).

The results of this study also indicate that the porcine *OPN* g.2442-2471indel significantly affected IMF content and ω3 FA levels. The porcine *OPN* g.2442-2471indel with 142 and 154-bp alleles seem to be the favorable alleles for IMF and ω3 FA deposition in the LT muscle tissues of Duroc pigs. Moreover, the porcine *OPN* g.3836A>G polymorphism was significantly associated with linolenic acid levels. Furthermore, the porcine *OPN* g.3836A>G with AG genotype was positively correlated with linolenic acid levels. Although, the role of the porcine *OPN* gene on IMF content and FA composition in muscle tissue was scarcely studied, the porcine *OPN* gene is an up-regulated expression in the LT muscle tissues of the porcine fetus (at 49-91 days post-conception). It has been reported that the *OPN* g.3836A>G polymorphism is related to myogenesis by regulating CCAAT/enhancer-binding protein beta (C/EBPβ) in pigs (Muráni et al., 2009). Moreover, the short interspersed nuclear element (SINE) variation in intron 6 of the porcine *OPN* gene is associated with backfat thickness and loin muscle area (Han et al., 2012). Additionally, the expression levels of the porcine *OPN* gene are related to IMF content in the LT muscle tissues. These pieces of evidence indicate that the *OPN* gene plays an important role in adipogenesis in skeletal muscle and adipose tissues (Han et al., 2012). The present study showed a significant association of the porcine *OPN* polymorphisms with IMF content and FA composition traits, especially with regard to linolenic and ω3 FA levels. Indeed, monogastric animals are not able to de novo synthesize a significant amount of essential FA, hence their diet would need to be supplemented (Zappaterra et al., 2020). Therefore, the observed genetic association could be indicated by an alteration in the gene that is related to absorption, transformation, or transport of these FAs resulting in modulation of the accumulated FAs in muscle tissue (Muñoz et al., 2012; Supakankul and Mekchay, 2016). All this information indicates that the *OPN* gene might be involved in lipid and FA deposition in the muscle tissue of pigs. In this study, our results indicated the effects of *OPN* polymorphisms on IMF content and FA composition in the LT muscle tissue of pigs. Further studies will be required to confirm the association of the *OPN* gene with IMF content and FA composition traits in various pig populations.

CONCLUSION

In the present study, we have identified the polymorphisms of the porcine *OPN* gene and elucidated their effects on IMF content and FA composition in the LT muscles of pigs. Two polymorphic sites of the porcine *OPN* gene (g.2442-2471indel and g.3836A>G) revealed an association with IMF content and FA composition of ω3 and linolenic acid levels. These results indicate that the *OPN* gene is essential for lipid deposition and FA accumulation in the muscle tissue of pigs. Thus, the porcine *OPN* gene may be used as candidate marker for the selection of IMF and FA deposition in the muscles of pigs.

CONFLICT ON INTEREST

The authors of this manuscript declare that they hold no conflicts of interest.

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AUTHORS CONTRIBUTION

Nanthana Pothakam; Investigation, data curation, writing - original draft.
Worrarak Norseeda; Investigation, data curation, writing - review and editing.
Guisheng Liu; Conceptualization, methodology, writing - review and editing.
Tawatchai Teltathum; Investigation, formal analysis, writing - review and editing.
Pantaporn Supakankul; Methodology, data curation, writing - review and editing.
Watcharapong Naraballobh; Data curation, formal analysis, writing - review and editing.
Trisadee Khamlor; Data curation, formal analysis, writing - review and editing.
Korawan Sringarm; Methodology, data curation, writing - review and editing.
Supamit Mekchay; Conceptualization, supervision, investigation, formal analysis, writing - original draft, writing-review and editing, project administration.

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