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Effect of *UBE3C* polymorphisms on intramuscular fat content and fatty acid composition in Duroc pigs

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ABSTRACT. Ubiquitin protein ligase E3C (*UBE3C*) is involved in the ubiquitin-proteasome pathway, and several ubiquitin protein ligases are important for fat deposition and lipid metabolism. The objective of this study was to analyze the association between a single nucleotide polymorphism (SNP) of the *UBE3C* gene with intramuscular fat (IMF) content and fatty acid (FA) composition in Duroc pigs. Four SNP markers (g.1586399A>G, g.1591358G>A, g.1600132G>C, and g.1600166G>A) of porcine *UBE3C* were genotyped using the polymerase chain reaction-restriction fragment length polymorphism method, and their associations with IMF content and FA composition were investigated in a commercial Duroc pig population. Two SNP

markers (g.1586399A>G and g.1591358G>A) were segregated among the pigs. No *UBE3C* polymorphisms at g.1600132G>C or g.1600166G>A were observed. The *UBE3C* g.1586399A>G SNP was significantly associated with IMF content, while the *UBE3C* g.1591358G>A SNP was associated with palmitic, stearic, eicosenoic, and eicosadienoic acid levels, and saturated FA levels. These results suggest that polymorphisms in porcine *UBE3C* are correlated with IMF content and FA composition, and confirm the importance of porcine *UBE3C* as a candidate gene for fat deposition in pigs.

Key words: *UBE3C*; Intramuscular fat; Fatty acid; Pig

INTRODUCTION

Intramuscular fat (IMF) content is an important determinant of meat quality in the pig (Gerbens et al., 2000; Sanchez et al., 2007; Lee et al., 2010b), and is positively correlated with quality characteristics such as juiciness, flavor, and tenderness (Fernandez et al., 1999; Gerbens et al., 1999, 2000; Zhao et al., 2012). Fatty acid (FA) composition serves as an indicator of lipid quality (Sanchez et al., 2007; Lee et al., 2010b). IMF content and FA composition are considered complex traits, and are controlled by genetic and environmental factors (Rosenvold and Andersen, 2003; Cesar et al., 2014). In order to understand the genetic control of IMF content and FA composition, many genomic regions and putative positional candidate genes that are associated with IMF deposition and FA composition have been identified as genetic dissection traits (Kim et al., 2011; Muñoz et al., 2013; Nonneman et al., 2013; Yu et al., 2013; Chen et al., 2014). Significant quantitative trait loci (QTLs) for fatness, including IMF content and FA composition, are located on SSC18 (Lee et al., 2003; Choi et al., 2010; Kim et al., 2011; Rückert et al., 2012; Uemoto et al., 2012; Muñoz et al., 2013). Several QTLs for IMF content and FA composition (C14:0, C16:0, C18:2, and C20:5) in muscle tissue and subcutaneous fat are located at position 1-26 Mb of SSC18 (Sanchez et al., 2007; Pérez-Montarelo et al., 2012; Ramayo-Caldas et al., 2012; Uemoto et al., 2012; Nonneman et al., 2013). Interestingly, within this region, several potential candidate genes for fat deposition traits are present, such as leptin (*LEP*), insulin-induced gene 1 (*INSIG1*), and ubiquitin protein ligase E3C (*UBE3C*). Previous studies have used *LEP* and *INSIG1* as candidate genes for determining the fat deposition and FA composition of meat in various mammal species (Li et al., 2003; Pérez-Montarelo et al., 2012; De Jager et al., 2013; Ramayo-Caldas et al., 2014; Toedebusch et al., 2014). However, information on the association between *UBE3C* and IMF deposition and FA composition is limited. The present study evaluated the effects of *UBE3C* on IMF deposition and FA composition in pigs.

Porcine *UBE3C* has been successfully characterized; its coding sequence is 1878 bp long and is composed of 11 exons and 10 introns, which encode a peptide of 492 amino acids (ENSSSCT00000017866; http://asia.ensembl.org/index.html). Porcine *UBE3C* polymorphisms have been documented in the Ensembl database (http://asia.ensembl.org/index.html). A total of 1489 single nucleotide polymorphisms (SNPs) of porcine *UBE3C* have been identified and classified, consisting of 10 missense, 11 synonymous, 1200 intron, 2 5'-untranslated region, 120 upstream, and 146 downstream variants. *UBE3C* is an E3 ubiquitin protein ligase, and is involved in ubiquitination (Rotin and Kumar, 2009). It plays a role in

the ubiquitin-proteasome pathway for protein degradation (Ponsuksili et al., 2009; Lee et al., 2010a; Pasaje et al., 2011). Recent evidence suggests that several E3 ubiquitin proteins play roles in fat deposition, lipid metabolism, and the inflammatory response in mammals (Qi et al., 2006; Pasaje et al., 2011; Song et al., 2013; Abe et al., 2014). E3 ubiquitin ligase COP1 is a key regulator of lipid metabolism and energy homeostasis in mice (Qi et al., 2006). E3 ubiquitin ligase MG53 and E3 ubiquitin ligase CBL-B induce insulin resistance in muscle and adipocyte tissue (Song et al., 2013; Abe et al., 2014). In addition, *UBE3C* exhibits altered expression levels in pig adipocyte tissue (Toedebusch et al., 2014), and E2 and E3 ubiquitin proteins are expressed in muscle and are associated with meat quality in pigs (Ponsuksili et al., 2010; Huynh et al., 2013). Therefore, E3 ubiquitin ligase can be regarded as a candidate gene for fat deposition in muscle. To elucidate the effects of this gene, porcine *UBE3C* polymorphisms were identified, and their associations with IMF content and FA composition were investigated in a commercial Duroc pig population.

MATERIAL AND METHODS

Animals and phenotypic measurements

The study was approved by the Animal Ethics Committee of the Faculty of Agriculture, Chiang Mai University, Thailand. A total of 324 pigs (188 gilts and 136 barrows) were obtained from a commercial Duroc population (Betagro Hybrid International Company, Thailand). All of the pigs were reared under commercial conditions and slaughtered at about 90 kg of body weight. Longissimus dorsi (LD) muscle samples from the 10th rib were collected and used to determine IMF content by the ether extraction method (AOAC, 2000). FA composition was measured by gas chromatography (SCION 456-GC, Bruker Daltonics Inc., Fremont, CA, USA) with an RT-2560 capillary column (Restek, Bellefonte, PA, USA). Individual FAs were calculated as a percentage of total FAs. The proportions of saturated (C14:0, C16:0, C18:0, and C20:0), monounsaturated (C16:1n-9, C18:1n-9, and C20:1n-9), polyunsaturated (C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6), ω3 (C18:3n-3, C20:5n-3, and C22:6n-3), ω6 (C18:2n-6, C18:3n-6, and C20:3n-6), and ω9 (C16:1n-9 and C18:1n-9) FAs were calculated.

Isolation of genomic DNA and SNP genotyping

The isolation of genomic DNA from muscle tissue was conducted using the phenol-chloroform method (Sambrook and Russell, 2001). The quantity and purity of the genomic DNA samples were measured using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to verify the presence and content of porcine *UBE3C* polymorphisms. Four polymorphic sites on porcine *UBE3C* were selected based on the restriction enzymes available in the Ensembl database, and consisted of g.1586399A>G (rs81329544), g.1591358G>A (rs32466023), g.1600132G>C (rs81261314), and g.1600166G>A (rs81261315). Specific primers were designed based on the porcine *UBE3C* nucleotide sequence (GenBank accession No. NC_010460), as shown in Table 1. A mismatched primer was designed to introduce a recognition site of the restriction enzyme for genotyping (Table 1). PCR amplifications were performed using 50 ng genomic DNA, 4 pM of each primer (Table 1), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 U *Taq* DNA polymerase (Fermentas), and 1X *Taq* buffer (20 mM

Tris-HCl, pH 8.4, 50 mM $(NH_4)_2SO_4$; Fermentas) in a final volume of 20 µL. The thermal cycling conditions were as follows: denaturation at 94°C for 3 min; 35 cycles at 94°C for 30 s, 58°-60°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 5 min. Digestions were performed using the restriction enzymes (Table 1), and the digested fragments were analyzed by 6% polyacrylamide gel electrophoresis.

Table 1. Primer sequences, polymerase chain reaction conditions, and restriction enzymes used for genotyping polymorphisms of the porcine *UBE3C* gene.

SNP	Primer sequence*	Size (bp)	Ta (°C)	Restriction enzyme
g.1586399A>G	F: 5'-AACCTGTCCTGTTGCTCTCA-3'	192	58	*Bsh*1236I
	R: 5'-CAGGAGAGCCAGCTAAACT-3'			
g.1591358G>A	F: 5'-CTATGCCCACCTTAGAAGGA-3'	319	58	*Hin*6I
	R: 5'-GCTTGCTGCTGCTCTTTTAG-3'			
g.1600132G>C	F: 5'-AGGAGAGGGGAGATGGCG-3'	126	60	*Hin*6I
	R: 5'-AACGCCTTCAGAAACCACC-3'			
g.1600166G>A	F: 5'-AGTCCATCTGCAATGTGAGC-3'	149	60	*Hpy*CH4V
	R: 5'-CAGAAACCACCGTTCAGATA-3'			

*Mismatched nucleotide is underlined to generate a recognition site of the restriction enzyme for genotyping. SNP, single nucleotide polymorphism; Ta, annealing temperature.

Statistical analysis

Genotypic and allelic frequencies were calculated, and the effects of porcine *UBE3C* on IMF content and FA composition were analyzed using a general linear model that included sex and marker genotype as fixed effects. Differences between the least square mean averages for the porcine *UBE3C* genotypes were considered, with the value of significance set at $P < 0.05$.

RESULTS

Verification of porcine *UBE3C* polymorphisms

Four SNP markers of porcine *UBE3C* were selected for verification in the commercial Duroc population. The results revealed that two SNP markers (g.1586399A>G and g.1591358G>A) on introns 5 and 6 were segregated among the Duroc pigs (Figure 1). There were no polymorphisms at g.1600132G>C or g.1600166G>A.

Genotypic and allelic frequencies of porcine *UBE3C*

The genotypic and allelic frequencies of porcine *UBE3C* were estimated, and the results are shown in Table 2. At the g.1586399A>G locus, two genotypes (AA and AG) were present, while at g.1591358G>A, three genotypes were observed. At the g.1600132 and g.1600166 loci, these two SNPs were fixed as g.1600132G and g.1600166G. A low frequency of the homozygous AA genotype (2%) at the g.1591358G>A locus was observed. Consequently, genotype AA was excluded from an analysis of the association between the g.1591358G>A SNP and fat deposition. Alleles of g.1586399A and g.1591358G were more frequently observed.

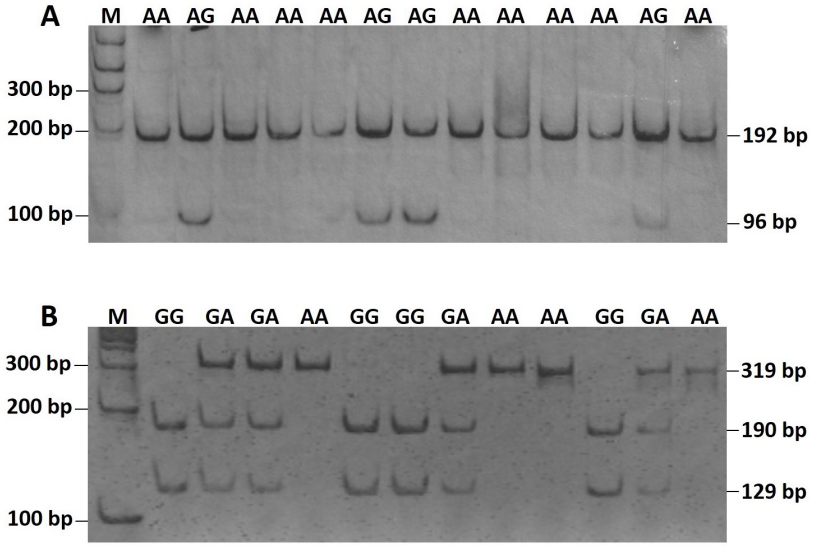

Figure 1. Polymerase chain reaction-restriction fragment length polymorphism analysis of the porcine *UBE3C* gene. **A.** Polymerase chain reaction fragments of g.1586399A>G and **B.** g.1591358G>A were digested using the restriction enzymes *Bsh*1236I and *Hin*6I, respectively. *Lane M*, 100-bp DNA ladder; *UBE3C* genotypes are given at the top of each lane.

Table 2. Genotypic and allelic frequencies of porcine *UBE3C* genotypes in Duroc pigs.

Marker		Genotypic frequency			Allelic frequency	
	N	AA	AB	BB	A*	B
g.1586399A>G	220	0.75	0.25	0	0.87	0.13
g.1591358G>A	324	0.78	0.20	0.02	0.88	0.12
g.1600132G>C	195	1	0	0	1	0
g.1600166G>A	208	1	0	0	1	0

*Allele A represents wild-type alleles of *UBE3C* g.1586399A, g.1591358G, g.1600132G, and g.1600166G for each locus and allele B represents mutant alleles of g.1586399G, g.1591358A, g.1600132C, and g.1600166A for each locus.

Associations between porcine *UBE3C*, IMF content, and FA composition

The effects of porcine *UBE3C* on IMF content and FA composition are shown in Tables 3 and 4. The g.1586399A>G SNP was significantly related to IMF content, and pigs with the AA genotype had a higher IMF content than pigs with the AG genotype ($P < 0.05$). There was no significant association between the g.1586399A>G SNP with any FA trait (Table 3). However, the g.1591358G>A SNP was significantly associated with FA composition in the LD muscle. Pigs with the GG genotype had significantly higher palmitic (C16:0), stearic (C18:0), eicosenoic (C20:1n-9), and eicosadienoic acid (C20:2n-6) levels and saturated FA levels than those with the GA genotype ($P < 0.05$). There was no significant association between the g.1591358G>A SNP and IMF content (Table 4).

Table 3. Associations between porcine *UBE3C* g.1586399A>G, IMF content, and FA composition in the longissimus dorsi muscle of Duroc pigs.

Trait (%)	Genotype (means ± SE)[a]		P value
	AA	AG	
IMF	5.963 ± 0.339[b]	4.169 ± 0.555[c]	0.0426
C12:0 (Lauric acid)	0.139 ± 0.021	0.105 ± 0.044	0.2910
C14:0 (Myristic acid)	1.480 ± 0.061	1.523 ± 0.130	0.7722
C16:0 (Palmitic acid)	14.754 ± 0.880	14.103 ± 1.853	0.7580
C18:0 (Stearic acid)	12.546 ± 0.602	11.050 ± 1.267	0.3036
C20:0 (Arachidic acid)	0.299 ± 0.045	0.274 ± 0.095	0.8155
Saturated FA	29.081 ± 1.414	26.951 ± 2.975	0.5310
C16:1n-9 (Palmitoleic acid)	4.386 ± 0.206	4.763 ± 0.433	0.4470
C18:1n-9 (Oleic acid)	34.699 ± 1.772	36.459 ± 1.989	0.2234
C20:1n-9 (Eicosenoic acid)	1.659 ± 0.129	1.574 ± 0.272	0.6974
Monounsaturated FA	40.780 ± 1.864	46.263 ± 3.921	0.2246
C18:2n-6 (Linoleic acid)	20.112 ± 1.336	18.834 ± 2.812	0.6902
C18:3n-6 (Linolenic acid)	0.093 ± 0.032	0.109 ± 0.044	0.1812
C20:2n-6 (Eicosadienoic acid)	1.702 ± 0.161	1.205 ± 0.240	0.0786
C20:3n-6 (Homolinolenic acid)	0.201 ± 0.081	0.189 ± 0.072	0.6042
C20:4n-6 (Arachidonic acid)	0.982 ± 0.141	0.119 ± 0.231	0.1710
Polyunsaturated FA	23.090 ± 1.450	21.456 ± 2.899	0.5505
ω3 FA	0.787 ± 0.099	0.578 ± 0.208	0.3830
ω6 FA	20.395 ± 1.332	19.177 ± 2.802	0.7033
ω9 FA	39.085 ± 1.793	41.222 ± 2.122	0.1976

[a]Means ± SE represent least square means ± standard error. [b,c]Values in each row with different superscript letters were significantly different ($P < 0.05$). IMF, intramuscular fat; FA, fatty acid.

Table 4. Associations between porcine *UBE3C* g.1591358G>A, IMF content, and FA composition in the longissimus dorsi muscle of Duroc pigs.

Trait (%)	Genotype (means ± SE)[a]		P value
	GG	GA	
IMF	5.095 ± 0.280	4.624 ± 0.591	0.4748
C12:0 (Lauric acid)	0.113 ± 0.018	0.166 ± 0.034	0.1652
C14:0 (Myristic acid)	1.471 ± 0.058	1.396 ± 0.110	0.5394
C16:0 (Palmitic acid)	15.629 ± 0.456[b]	13.554 ± 0.859[c]	0.0350
C18:0 (Stearic acid)	14.172 ± 0.410[b]	12.120 ± 0.771[c]	0.0209
C20:0 (Arachidic acid)	0.290 ± 0.040	0.270 ± 0.076	0.8136
Saturated FA	31.562 ± 0.984[b]	27.340 ± 1.186[c]	0.0155
C16:1n-9 (Palmitoleic acid)	4.550 ± 0.178	4.355 ± 0.335	0.6028
C18:1n-9 (Oleic acid)	37.292 ± 1.636	34.872 ± 2.087	0.1198
C20:1n-9 (Eicosenoic acid)	1.976 ± 0.157[b]	1.212 ± 0.296[c]	0.0246
Monounsaturated FA	43.818 ± 1.738	40.439 ± 2.079	0.0856
C18:2n-6 (Linoleic acid)	18.712 ± 1.296	20.384 ± 2.437	0.5397
C18:3n-6 (Linolenic acid)	0.098 ± 0.030	0.063 ± 0.026	0.1798
C20:2n-6 (Eicosadienoic acid)	1.683 ± 0.139[b]	1.124 ± 0.233[c]	0.0398
C20:3n-6 (Homolinolenic acid)	0.241 ± 0.072	0.186 ± 0.135	0.7131
C20:4n-6 (Arachidonic acid)	0.974 ± 0.232	1.207 ± 0.312	0.1878
Polyunsaturated FA	21.708 ± 1.369	22.964 ± 2.463	0.4103
ω3 FA	0.690 ± 0.092	0.822 ± 0.172	0.4947
ω6 FA	19.065 ± 1.296	20.493 ± 2.438	0.6004
ω9 FA	41.842 ± 1.614	39.227 ± 2.113	0.1126

[a]Means ± SE represent least square means ± standard error. [b,c]Values in each row with different superscript letters were significantly different ($P < 0.05$). IMF, intramuscular fat; FA, fatty acid.

DISCUSSION

Ubiquitination, a process in the ubiquitin-proteasome system, is responsible for the majority of protein degradation in the intracellular pathway of mammalian cells (Ponsuksili et al., 2009; Keller et al., 2012; Bugliani et al., 2013). Three enzymatic components, designated as E1 (activation enzyme), E2 (conjugate enzyme), and E3 (ligase enzyme), promote the conjugation of ubiquitin molecules to the target proteins (Keller et al., 2012; Bugliani et al., 2013). The ubiquitin-proteasome named 26S proteasome is a large multicatalytic protease complex that degrades ubiquitinated proteins to small peptides (Keller et al., 2012).

Previous studies have shown that gene expression in the ubiquitination system is associated with meat quality (Ponsuksili et al., 2008, 2009), and ubiquitin protein ligase *UBE3B*, a paralog of *UBE3C*, is significantly associated with carcass, water-holding capacity, meat color, and conductivity traits in pigs (Basel-Vanagaite et al., 2012; Huynh et al., 2013; Jiang et al., 2014). Many studies have reported that E3 ubiquitin protein ligases are involved in fat deposition, lipid metabolism, energy homeostasis, insulin resistance, and immune responses in mammals (Qi et al., 2006; Pasaje et al., 2011; Song et al., 2013; Abe et al., 2014). Although there is little published information regarding *UBE3C* and its possible functions related to fat deposition in muscle, it is of significant interest as a positional candidate gene for fat deposition in muscle due to its location on SSC18, near the QTLs for IMF content and FA composition (Pérez-Montarelo et al., 2012; Ramayo-Caldas et al., 2012; Uemoto et al., 2012).

In the present study, we verified polymorphisms of porcine *UBE3C* and evaluated the effects of this gene on IMF content and FA composition in LD muscle. Two of four SNPs (g.1586399A>G and g.1591358G>A) of porcine *UBE3C* were segregated in the Duroc pigs, and a high frequency of the alleles g.1586399A and g.1591358G was observed. The results indicate that there has been strong selection pressure on desirable production traits that are associated with the *UBE3C* g.1586399A and g.1591358G alleles. These two SNPs were significantly associated with IMF content and FA composition; the g.1586399A>G SNP was significantly associated with IMF content, while the g.1591358G>A SNP was significantly associated with FA composition (palmitic, stearic, eicosenoic, and eicosadienoic acids, and saturated FA). In addition, the favorable g.1586399A and g.1591358G alleles exhibited positive relationships with IMF and FAs in muscle. However, these two SNPs were identified in non-coding regions of porcine *UBE3C*. We hypothesize that these SNPs might be in linkage disequilibrium with other causal polymorphisms, which may be located in another region of *UBE3C*. Recent evidence suggests that *UBE3C* expression levels are downregulated in the adipocyte tissue of obese pigs (Toedebusch et al., 2014). This result is consistent with those obtained from a previous study, which found that E3 ubiquitin ligase CBL-B deficiency increases macrophage activation and accumulation in adipose tissue, resulting in induced insulin resistance in mice (Abe et al., 2014). In contrast, upregulated E3 ubiquitin ligase MG53 causes metabolic syndrome that includes insulin resistance, obesity, hypertension, and dyslipidemia in mice (Song et al., 2013). E3 ubiquitin ligase COP1 is associated with the pseudokinase TRB3 in deactivating acetyl-coenzyme A carboxylase, which is a key regulatory enzyme in the FA synthesis pathway (Qi et al., 2006). This indicates that E3 ubiquitin protein ligases are key regulators of fat deposition and energy homeostasis. Our results demonstrate that porcine *UBE3C* polymorphisms are correlated with IMF content and FA composition. Further studies will be required to test these SNPs in variant populations of pig breeds, in order to confirm the association with fat deposition. In addition, the association between porcine

UBE3C SNPs and transcriptional and protein expression levels in adipocyte and muscle tissue needs to be examined, in order to gain a better understanding of the molecular mechanisms underlying *UBE3C* function in fat deposition in muscle.

In conclusion, we verified variant DNA markers in porcine *UBE3C* and analyzed the effects of porcine *UBE3C* on IMF content and FA composition in pigs. Two porcine *UBE3C* polymorphisms (g.1586399A>G and g.1591358G>A) were associated with IMF content and FA composition. These results confirm the importance of porcine *UBE3C* in fat deposition in muscle. Therefore, these two SNPs may be used as candidate markers for the genetic improvement of fat deposition in pigs.

Conflicts of interest

The authors declare no conflict of interest.

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