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Genomic characterization of the porcine *CRTC3* and the effects of a non-synonymous mutation p.V515F on lean meat production and belly fat

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ABSTRACT

cAMP-responsive element-binding protein (CREB)-regulated transcriptional coactivator 3 (CRTC3) is well known to be related to obesity in humans and mice. However, the effects of *CRTC3* have not been studied in pigs. Here, we characterized the structure of the porcine *CRTC3* gene and identified single nucleotide polymorphisms (SNPs) in its coding region. Moreover, mRNA expression profiles of *CRTC3* in muscle and fat tissues were examined. Of the 40 identified SNPs, the p.V515F mutation, located on exon 16, was genotyped in 368 Yorkshire pigs. The p.V515F mutation was significantly associated with lean meat production ability, including reduced back fat thickness ($P = 0.0317$) and loin eye area ($P = 0.0174$). Moreover, the SNP was significantly associated with differences in intermuscular fat ($P = 0.0092$), total muscle area in the belly ($P = 0.0108$), and total fat percentage in the belly ($P = 0.0298$). Taken together, our results suggest that the p.V515F mutation affects to lean meat production ability and amount of belly fat.

1. Introduction

The pig is one of the most common livestock animals for meat (USDA/ERS, 2017). In Asia, pork belly is the most popular cut of pork because of Asian food culture (Marcoux, Pomar, Faucitano, & Brodeur, 2007; Nam, Jo, & Lee, 2010). However, pork belly has a higher fat composition compared to other cuts such as loin and ham; consumers tend to avoid higher fat content in belly meat for health reasons (Seman, Barron, & Matzinger, 2013; Smith, West, & Carpenter, 1975; Trusell et al., 2011). Moreover, consumers have a tendency to prefer leaner bellies, because the excess fatty bellies have been problematic in South Korea (Choe, Yang, Lee, & Go, 2015; Park et al., 2013). Pork belly has been improved to reduce fat, but it still has much fat (Choe et al., 2015). Therefore, it is of great importance to produce low-fat and lean belly meat in pigs. Many environmental factors, such as diet and the gut microbiome, are known to affect fatty acid accumulation in fat tissues (Bäckhed et al., 2004; Kasai et al., 2003; Kobori, Masumoto, Akimoto, & Oike, 2011; Ley, Turnbaugh, Klein, & Gordon, 2006; Stewart et al., 2008; Turnbaugh et al., 2006). There has been considerable research on breeding herds to identify major genes and quantitative trait loci (QTLs) associated with fat deposition, intramuscular fat and back fat thickness (BFT) (Cho, Kim, Jeon, & Chung, 2011; Guo et al., 2009; Milan et al., 2002; Sanchez et al., 2006; Urban, Mikolasova, Kuciel, Ernst, & Ingr, 2002; Zappaterra et al., 2016).

The cAMP-response element binding protein (CREB)-regulated transcriptional coactivator (CRTC) family regulates metabolic activity in the mitochondria (Altarejos et al., 2010). The CRTC family is known to play a key role in stimulating CREB activity upon its dephosphorylation and translocation to the nucleus (Screaton et al., 2004). Moreover, a previous study reported that CREB and CRTCs can lead to the upregulation of peroxisome proliferator-activated receptor-gamma coactivator-1A (PGC-1α) expression and induce mitochondrial biogenesis (Zhou et al., 2006). PGC-1α regulates the myocyte enhancer factor-2 (MEF2) transcription factor, which plays a role in muscle fiber specification (Lin, Handschin, & Spiegelman, 2005). Therefore, CRTC is a core regulatory factor in energy metabolism (Song et al., 2010).

Of the genes in the CRTC family, previous studies have indicated that *CRTC3* plays an important role in regulating energy metabolism and the development of obesity (Liu, Xu, Wu, Wang, & Shan, 2017; Song et al., 2010; Than, Lou, Ji, Win, & Kaplowitz, 2011). *CRTC3* is highly expressed in adipose tissues and is known to lead to fat deposition via the regulation of insulin resistance and energy metabolism (Song et al., 2010). In human, *CRTC3* is associated with metabolic diseases such as type 2 diabetes and obesity (Lee, Lin, Hsieh, Wu, & Wu, 2014; Ou et al., 2014; Prats-Puig et al., 2016). Fatty acid deposition in abdominal subcutaneous fat can directly affect the fat composition of

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the belly. The physical location of *CRTC3* was found to be in the 50 to 75 cM region of chromosome 7, which has been reported as a QTL for abdominal fat composition (Guo et al., 2009). Therefore, we hypothesized that the function of *CRTC3* in pigs may also have important roles in fat metabolism and deposition in abdominal subcutaneous adipose tissue. However, no studies have been performed on pigs to characterize the genomic structure of *CRTC3* and its mutations; furthermore, no attempt has yet been made to identify its function in pigs.

Here, we characterized the genomic structure of and variations in the *CRTC3* gene and investigated the mRNA expression profiles of *CRTC3* in fat and muscle tissues. The aim of this study was to identify the effects of *CRTC3* on lean and low-fat meat production.

2. Materials and methods

2.1. Animals and trait measurements

A total of 368 castrated male Yorkshire pigs were used in this study. They were fed the same commercial diet from seven different farms belonging to one company. The pigs were slaughtered in twenty-four batches by a commercial abattoir according to standard procedures under the supervision of the Korean grading service for animal products. The carcasses were chilled at 4 °C for 24 h and the (LT) muscle was used to evaluate muscle traits. BFT was measured at the eleventh and final thoracic vertebrae, and the mean was calculated. The loin eye area (LEA) was measured at the last rib. The lightness of the meat was measured at the eighth and ninth thoracic vertebrae with a chromometer (CR-300; Minolta Camera Co., Japan). Marbling scores, determined by the National Pork Producers Council (NPPC) score guide, were obtained in the loin muscle. Pork belly meat was taken from a meat processing enterprise 24 h after slaughter. The belly was sliced to 15-mm thickness. Sliced belly meat from the sixth thoracic vertebra (Fig. 1) was scanned using a colour scanner (C3360; Canon, Japan). The total fat area (TFA) and total muscle area (TMA) were measured from scanned images using Image-Pro Plus software (Media Cybernetics, USA) (Fig. 1). Intermuscular fat thickness (IFT) was measured between the last cutaneous muscle and latissimus dorsi muscle in the first belly cross-section using a ruler (Fig. 1). All the measured traits in this study are presented as the mean with standard deviation and overall range (Table S1).

2.2. Identification of genomic sequence variations

To identify polymorphisms between or within four different pig breeds (Yorkshire, Landrace, Duroc and Berkshire; $n = 20$ per breed), direct sequencing analysis was performed using porcine-specific primers on the coding regions of the *CRTC3* gene (accession No. ENSSSCT00000029592). The primer sets were designed using Primer3 software (Table S2). Genomic DNA was extracted from EDTA-treated blood using the G-DEX IIb Genomic DNA Extraction Kit (Intron Biotechnology, Korea) from a total of 80 pigs from the above four breeds (Yorkshire, Landrace, Duroc, Berkshire). PCR was performed using a Mastercycler gradient (Eppendorf Co., Germany) at a total volume of 20 μL, including 100 ng porcine genomic DNA template, 10 pmol of each primer, 0.25 mM of each dNTP, 1 × PCR buffer, and 1.25 U DNA polymerase (i-Max II; Intron biotechnology, Korea). Sequencing analysis was performed using an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA). Sequence assembly and polymorphism identification was executed using SeqMan.

2.3. Genotyping of p.V515F

Genotyping was performed on the p.V515F locus. We used 368 castrated male Yorkshire pigs as the study population. Genomic DNA was extracted from LT muscle tissue using a DNA isolation kit (G-Dex IIc; Intronbio, Korea). The p.V515F genotypes were identified using a PCR-restriction fragment length polymorphism (RFLP) assay. PCR was performed using the same conditions described in 2.2. The amplification conditions were as follows: 94 °C for 10 min, followed 30 cycles of 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR products were digested using *Hinc*II restriction enzyme at 37 °C for 1 h for RFLP analysis. The restriction patterns are presented in Fig. 2.

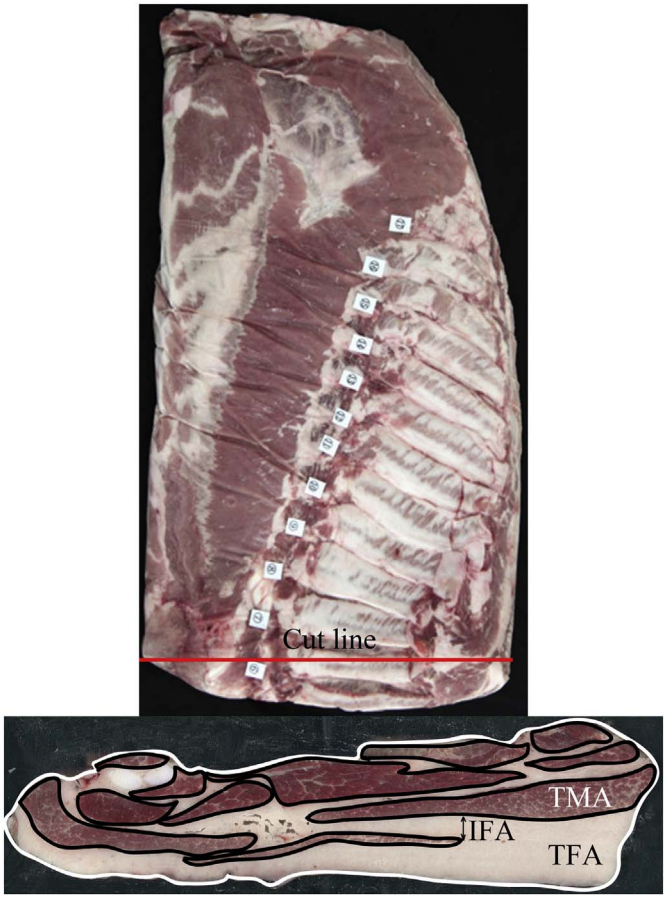

Fig. 1. Belly dissection and measurement regions. The red line shows a cross-section of the sixth thoracic vertebra. TMA, total muscle area (cm^2); TFA, total fat area (cm^2); IFT, intermuscular fat thickness (mm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Quantitative real-time PCR (qPCR) analysis

For the mRNA expression analysis, LT muscle and back fat (BF) tissues were collected from carcasses 45 min after slaughter and stored in liquid nitrogen. Total RNA was isolated from 10 mg muscle tissue using TRIzol reagent (Invitrogen, USA), or from 20 mg fat tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Netherlands). Next, 2 μg total RNA was mixed in DEPC-treated water at a final volume of 7.5 μL. Thereafter, 1 μL 10 × buffer and 1.5 μL DNase were added to the mixture. The mixture was incubated at room temperature (RT) for 15 min, followed by the addition of 1 μL EDTA and incubation at 65 °C for 10 min using a Thermocycler (Eppendorf Co.). After DNase treatment, 1 μL dNTP (100 mM), 0.2 μL random primers, and 0.3 μL DEPC-treated water were added to the mixture. The mixture was incubated at 65 °C for 5 min, then placed in an ice bath for 2 min. Next, 4 μL 5 × First strand buffer, 2 μL DTT (0.1 M), 0.5 μL RNase inhibitor (40 U/μL), and 1 μL M-MLV reverse transcriptase (200 U/μL; Invitrogen) were added and the mixture was incubated at 37 °C for 2 min, 25 °C for 10 min, 37 °C for 90 min, and 70 °C for 15 min on a Mastercycler gradient. Synthesized cDNA samples were stored at − 20 °C. The mRNA