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Short communication

Polymorphism of the porcine miR-30d is associated with adipose tissue accumulation, its fatty acid profile and the *ME1* gene expression

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ABSTRACT

The miR-30 gene family includes potential regulators of adipogenesis. We searched for polymorphism in this gene family in 4 pig breeds: Duroc, Pietrain, Polish Landrace (PL), Polish Large White (PLW) and a synthetic line (L990). Altogether 5 single nucleotide polymorphisms (SNPs) in 3 genes were found, including one already known SNP rs340704946 within the pre-miR-30d genomic sequence. An association of the rs340704946 with intramuscular fat (IMF) content, abdominal fat accumulation and daily body mass gain in L990 was found. Moreover, the AG genotype was associated with an increased content of monounsaturated fatty acids (MUFA) in subcutaneous ($P=0.027$) and visceral fat ($P=0.007$) tissues when compared to the GG genotype. Analysis of PLW pigs revealed an association of the rs340704946 with transcript level of the *ME1* gene in longissimus dorsi (LD) muscle ($P=0.002$), as well as the *ME1* protein product in the LD muscle ($P<0.001$) and subcutaneous fat ($P<0.001$). We conclude that the rs340704946 can be considered as a functional polymorphism for pig production traits and fatty acid profile in adipose tissue.

1. Introduction

The miR-30 gene family includes potent regulators of human adipogenesis (Peng et al., 2014). Up-regulation of the miR-30 genes promotes adipogenesis of adipose tissue-derived stem cells, whereas inhibition of its expression blocks this process (Zaragosi et al., 2011). It was also observed that miR-30d gene is down-regulated in obese patients (Perri et al., 2012).

According to the miRBase and pigQTLdb databases, the pig miR-30 family genes are located in three autosomes: SSC1 (miR-30a, miR-30c2), SSC4 (miR-30b, miR-30d) and SSC6 (miR-30c1, miR-30e), within the QTL regions for longissimus dorsi (LD) muscle area, backfat thickness, average daily gain and intramuscular fat (IMF) content (Kozomara and Griffiths-Jones, 2014; Zhi-Liang et al., 2013).

In silico study suggests that the miR-30 family downregulates the *ME1* gene. The *ME1* gene encoding the malic enzyme is expressed in the adipose tissue and involved in fatty acid metabolism (Ramírez et al., 2014). Deep sequencing demonstrated co-expression of the miR-30 and *ME1* genes in porcine adipose and muscle tissues (Nielsen et al., 2010; Li et al., 2012). It was also reported that a polymorphism within the 3'UTR of the *ME1* gene may alter *ME1* transcript level (Bartz et al., 2014).

We hypothesized that a regulatory mechanism of porcine fatness involves miR-30 gene family and polymorphism within the genes may influence fatness via its effect on the *ME1* gene expression. Thus, the aim of the present study was searching for polymorphism in the porcine miR-30 gene family and its association with production traits, fatty acid (FA) profile in fat depots and the *ME1* expression.

2. Materials and methods

2.1. Animals and phenotypes

Three sample sets of gilts were included in this study (Table 1). All gilts were reared under the same conditions at a local Pig Testing Station, fed individually ad libitum with a commercial feed mix and slaughtered at 100 kg of body weight. The carcasses were dissected and the following traits were measured: backfat thickness (mm), abdominal fat weight (kg), lean meat content (%) and IMF content (%). Backfat thickness was measured with a ruler at 7 points: over the shoulder, over the back, at sacrum points I–III and at points C1 and K1 (on the vertical line extending from the height and a side edge of the LD muscle, respectively). These measurements were treated separately as 7 different phenotypic traits. IMF content was measured using the SOXTEC AVANTI 2050 automatic extraction

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Table 1
Sample sets of gilts used in this study.

Sample set	Breed	Relationship	Type of analysis
A	**Duroc** ($n=22$), **Pietrain** ($n=16$), **Polish Landrace** ($n=22$, excluded from the "b" analysis), **Polish Large White** (PLW, $n=25$)	Gilts shared no common sires or dams.	(a) Screening for new polymorphism ($n=85$) (b) Association between SNP rs340704946 and fatty acid profile in 3 tissues ($n=54$, excluding Polish Landrace, AA genotype and outliers)
B	**Polish Large White** ($n=205$), **synthetic line 990** ($n=233$)	Gilts of Polish Large White descended from 46 sires and 127 dams. Gilts of synthetic line 990 descended from 49 sires and 155 dams. Within the 205 PLW gilts 25 were from sample set A.	(c) Association between SNP rs340704946 in miR30d and production traits ($n=438$)
C	**Polish Large White** ($n=20$)	Subsample of Polish Large White from "B" sample set. Gilts shared no common sires or dams.	(d) Association between SNP rs340704946 and *ME1* transcript level in 2 tissues ($n=20$) (e) Association between SNP rs340704946 and ME1 protein level in 2 tissues ($n=20$)

system (Foss Tecator, Hoganas, Sweden) and analyzed in the log-transformed scale to reduce skewness. Moreover, two performance traits were considered: post-weaning average daily gain (calculated for the fattening period from 25 to 100 kg live weight) and feed conversion ratio (calculated as a ratio of the consumed feed and gain during the fattening period). Data on the FA profiles in subcutaneous fat, visceral fat and the LD muscle for this pig population were derived from our earlier studies (Bartz et al., 2014; Klensporf-Pawlik et al., 2012). The study was accepted by a local Bioethical Commission for Animal Care and Use in Poznan (Poland).

2.2. Methods

Sequencing procedure described earlier by Bartz et al. (2014) was applied. Six primer pairs were designed using the Primer3Plus tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to cover fragments of all miR-30 genes in the pig genome (Supplementary Table 1). PCR conditions were as follows: preliminary denaturation at 94 °C for 5 min, 36 cycles of 94 °C for 30 s (denaturation), 58–64 °C for 30 s (primer annealing at a temperature specific for the analyzed fragment), 72 °C for 30 s (extension), and the final extension at 72 °C for 10 min. The reaction mix consisted of 50 μM DNA, 0.25 mM of each primer, 0.125 mM of each dNTP, and 0.75 U of Taq Polymerase (EURx, Poland) in a total volume of 20 μl. Amplification was performed in a PTC-200 thermocycler (MJ Research, Watertown, MA).

Genotyping of the g.33458A > G polymorphism (rs340704946) in the miR-30d gene was performed by a PCR-RFLP test using the *Hinf*I endonuclease (New England Biolabs, Germany) that recognizes exclusively the wild-type sequence containing the reference nucleotide (NEBcutter 2.0, http://tools.neb.com/NEBcutter2). The digestion reaction was prepared according to the manufacturer's protocol. Restriction endonuclease products were separated in 2% agarose gel.

The samples of LD muscle and subcutaneous adipose tissue were collected immediately after slaughter, snap-frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from samples using the TriPure Isolation Reagent (Roche, Warsaw, Poland) according to the manufacturer's protocol. The RNA quantity and purity were measured using NanoDrop 2000 (Thermo Scientific, Waltham, MA) and RNA integrity was checked on agarose gel. For cDNA synthesis 2 μg of RNA were reversely transcribed with the use of the Transcriptor High Fidelity cDNA Synthesis kit (Roche, Warsaw, Poland). The real-time PCR was performed on a LightCycler 480 II (Roche, Warsaw, Poland) based on the SYBR Green detection system. The cyclophilin A (PPIA) and β-actin (ACTB) genes were used as references for the normalization of data. Real-time PCR primers were as followed: ME1-F: 5' CCACCTTGCTTCATCAGTCA; ME1-R: 5' GCAAGCCAGACCCACAGT; (198 bp); PPIA-F: 5' CACAAACGGTTCCCAGTTTT; PPIA-R: 5' TGTCCACAGTCAGCAATGGT; (171 bp); ACTB-F: 5' CCCCCGACACCAGGGCGTGAT; ACTB-R: 5' CGGCCAGAGGCGTACAGGGACAG; (330 bp). The relative quantification of the mRNA level was performed in duplicates based on the Second Derivative Maximum Method. Standard curves were designed as 10-fold dilutions of the PCR product. The abundance of the ME1 transcripts was then normalized to a geometric mean of the PPIA and ACTB reference genes (Vandesompele et al., 2002).

To perform Western blotting of ME1 product the frozen tissue samples were disrupted with a handheld rotor-stator homogenizer (TissueRuptor, Qiagen) and lysed in a buffer containing 60 mM TRIS-base, 2% SDS, 10% sucrose, and 2 mM PMSF. A total of 30 μg of protein was diluted in a sample buffer containing 2-mercaptoethanol, denatured for 5 min and separated by 12% SDS–polyacrylamide gel electrophoresis (5% stacking/12% resolving gel). After electrophoresis the proteins were electrotransferred onto a nitrocellulose membrane (Sigma). All immunodetection steps were performed on a SNAP id protein detection system (Millipore) in PBS buffer containing 0.25% nonfat milk and 0.1% Tween 20, while the membranes were washed in PBS/Tween. For ME1 and GAPDH detection the blots were probed with the primary rabbit anti-ME (1:1000, Abcam) and mouse anti-GAPDH (1:5000, Millipore) antibodies, respectively, and subsequently probed with HRP-conjugated secondary antibodies (1:500, Sigma). The immunoreaction was detected using Western Bright Quantum (Advansta). The protein amounts were quantified using the GeneSnap (Syngene) software.

To study the association between the rs340704946 and production traits we applied linear mixed models that included fixed effects of the SNP (3 levels: AA, AG, GG), the C1843T polymorphism at the *RYR1* locus (2 levels: CC or CT), age and carcass weight at slaughter as covariables, and sire as a random effect. The fixed effects of *RYR1* and covariables were excluded from the model if occurred non-significant. The two covariables were excluded from the models for average daily gain and food conversion ratio. Sires were treated as independent with equal variance. An additional relationship due to dams was ignored. The analysis of the effect of the SNP rs340704946 on FA content was performed for visceral fat, subcutaneous fat and LD muscle, separately for saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The statistical model included fixed effects of the rs340704946 (2 levels: AG, GG) and breed (3 levels: Duroc, Pietrain, PLW, Table 1). Between-breed differences in fatty-acid content recorded for this population were previously described by Klensporf-Pawlik et al. (2012). The statistical linear model for *ME1* transcript level included the fixed effect of the SNP (rs340704946, 3 levels: AA, AG, GG) and also the effect of the SNP c.*488A > G in the pig *ME1* gene (3'UTR, 2 levels: AA and AG). The SNP c.*488A > G was