Analysis of allele-specific expression of seven candidate genes involved in lipid metabolism in pig skeletal muscle and fat tissues reveals allelic imbalance of ACACA, LEP, SCD, and TNF

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Abstract

Analysis of allele-specific expression may help to elucidate the genetic architecture of complex traits including fat deposition in pigs. Here, we used pyrosequencing to investigate the allele proportions of candidate genes (ACACA, ADIPOR1, FASN, LEP, ME1, SCD, and TNF) involved in regulation of lipid metabolism in two fat deposits (subcutaneous and visceral fat) and longissimus dorsi muscle of pigs representing Polish Large White, Polish Landrace, Duroc, and Pietrain breeds. We detected differential allelic expression of ACACA, LEP, SCD, and TNF in all tissues analyzed. To search for putative cis-regulatory elements involved in allele-specific expression, we quantified the methylation level within CpG islands located in 5′-flanking regions of ACACA and SCD. Comparison between samples showing markedly disproportionate allelic expression and control groups with similar levels of both alleles did not reveal significant differences. We also assessed the association of rs321308225 (c.*195C>A) an SNP located in the 3′UTR of ACACA with its allelic expression in Polish Landrace pigs, but it was not significant. We conclude that allelic imbalance occurs frequently in regard to genes involved in regulation of lipid deposition in pigs, and further studies are necessary to identify cis-regulatory elements affecting ACACA, LEP, SCD, and TNF expression in porcine fat tissues and skeletal muscle.

Keywords Adipose tissue · Allele-specific expression · CpG methylation · Fatness · Pig · Skeletal muscle

Introduction

Preferential expression of one particular allele rather than the other is a common phenomenon across tissues and species (Chamberlain et al. 2015; Gaur et al. 2013). Such allelic imbalance can be a consequence of functional DNA polymorphisms or epigenetic factors including DNA methylation, chromatin modifications, nuclear positioning, or noncoding RNAs that affect gene expression in a cis-acting manner (Font-Cunill et al. 2018; Gaur et al. 2013; Takizawa et al. 2008). Analysis of allele-specific expression may be helpful in revealing the genetic basis of complex traits such as predisposition to obesity in humans and immunity traits in pigs (Knowles et al. 2017; Maroilley et al. 2017).

The modern pig industry is focused on efficient production and high meat quality. Adipose tissue accumulation is an important production trait in pigs that is affected by a combination of environmental and genetic factors, including the variation of gene expression in adipocytes and skeletal muscles (Stachowiak et al. 2016; Switonski et al. 2010). The analysis of allelic imbalance may facilitate the elucidation of genetic or epigenetic determinants of porcine fatness and improve the understanding of human obesity.

Here, we investigated the allelic expression of seven candidate genes involved in the regulation of lipid metabolism in fat deposits and skeletal muscle sampled from commercial pig breeds and attempted to decipher the effects of putative cis-regulatory elements on allelic imbalance of ACACA and SCD.

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Materials and methods

One hundred forty-two gilts representing Polish Large White (PLW; n = 48), Polish Landrace (PL; n = 35), Duroc (n = 38), and Pietrain (n = 21) breeds were reared under similar environmental conditions, fed ad libitum with the same commercial mix fodder, slaughtered at 100–105-kg weight, and dissected at the Pig Testing Station in Pawlowice (Poland). Peripheral blood, longissimus dorsi (l. dorsi) muscle, subcutaneous, and visceral fat tissues were collected.

Genomic DNA (gDNA) was extracted from blood, and exonic reporter SNPs (rSNPs) in ACACA (acetyl-Coa carboxylase alpha): rs81303284 (c.*99A>T); ADIPOR1 (adiponectin receptor 1); rs81508987 (c.759G>A); FASN (fatty acid synthase): rs324640280 (c.339C>T); LEP (leptin): rs45431504 (c.289T>C); ME1 (malic enzyme 1): rs328566530 (c.582T>C); SCD (stearyl-Coa desaturase): rs334462984 (c.*931C>G); and TNF (tumor necrosis factor): rs80945725 (c.306A>G) were genotyped by capillary sequencing on a 3130 Genetic Analyzer (Applied Biosystems).

RNA was extracted from subcutaneous, visceral fat and l. dorsi muscle. Prior to reverse transcription, RNA samples were digested with DNase I to remove contaminating gDNA. Allele quantification assays were designed with the use of PyroMark Assay Design 2.0 software (Qiagen). All primers obtained high-quality scores and only primers annealing to a DNA template without mutation sites were used. Allele proportions for each rSNP in cDNA and gDNA were measured by pyrosequencing of cDNA and gDNA using Pyromark Q48 Autoprep system (Qiagen). Allelic ratios were calculated by dividing the percentage of one allele by the other. Any bias resulting from variations in nucleotide incorporation during pyrosequencing reaction was normalized for each gene by dividing the allelic ratio of cDNA and gDNA samples by the mean allelic ratio from gDNA. The bi-directional character of ASE was neutralized by dividing the higher percentage by the lower, as described by Olbrormski et al. (2013). Next, the allelic transcript ratios were log_{10} transformed and the mean allelic expression between cDNA and gDNA for each breed and tissue was compared by two-tailed t test with unequal variances (Forton et al. 2007; Murani et al. 2009). The methylation status was determined for CGP islands (CGI) localized in 5′-flanking regions of ACACA (chromosome 12; CGI1: 38,874,595...38,875,705 and CGI2: 38,824,323...38,825,369) and SCD (chromosome 14: 111,460,649...111,461,395) based on porcine genome data (Sscrofa11.1, NC_010454.4). Genomic DNA was isolated from visceral fat and skeletal muscle and bisulfite-converted. 5-Methylcytosine (5-mC) levels (%) were quantified using Pyromark Q48 Autoprep pyrosequencer (Qiagen) and compared with Mann-Whitney Rank Sum Test between samples showing similar (n = 10 for ACACA in visceral fat; n = 10/9 for SCD in visceral fat/l. dorsi muscle) and extremely imbalanced allelic expression (n = 7 for ACACA in visceral fat; n = 5/9 for SCD in visceral fat/l. dorsi muscle).

Candidate SNPs in ACACA: rs321308225 (c.*195C>A) and TNF: rs328373700 (c.-791C>T) were genotyped using capillary sequencing. Due to the genotype distribution, association between analyzed SNP and allelic transcript expression was performed for rs321308225 in the PL breed only, using a two-tailed t test with unequal variances for log_{10} transformed allelic transcript ratios of homozygous versus heterozygous samples, as described by Murani et al. (2009).

All primers used in the study are listed in Suppl. Table S1. For a detailed description of methods, please see Stachowiak et al. (2018).

Results and discussion

We first identified animals that were heterozygous for an exonic reporter SNP (rSNP) in each gene, which is necessary to distinguish allelic transcripts and quantify their proportions. Based on the frequencies of heterozygous genotypes in our pig populations (Suppl. Table S2), measurements of allelic ratios were performed in breeds where at least 10 heterozygotes were available, i.e., in PLW, PL, Duroc, and Pietrain for ACACA; PLW and PL for ADIPOR1, FASN, and TNF; PLW and Duroc for LEP and SCD; and PLW, PL, and Duroc for ME1 (Fig. 1, Suppl. Fig. S1). Pyrosequencing was used as a reliable and sensitive means of studying allele-specific expression (Wang and Elbein 2007). We detected allelic imbalance of ACACA, LEP, SCD, and TNF in all breeds and tissues analyzed (Table 1). The most significant differences (p < 0.001) were found for ACACA in all tissues, for LEP in skeletal muscle, and for TNF in visceral fat but the effects were breed-specific (Table 1). The bi-directional nature of allelic imbalance of ACACA, LEP, SCD, and TNF (Fig. 1) provides evidence that the regulatory elements that affect their allelic expression are not in linkage disequilibrium with exonic rSNPs used to quantify allelic proportions. Mean allelic ratios calculated for each breed and tissue showed that the same allele was overexpressed for ACACA (A allele), LEP (C allele), and SCD (G allele) in all groups tested and for TNF (A allele) in PLW breed. Interestingly, this overrepresentation of one allele was also related to its higher frequency in several breeds and the highest effect was observed in case of allelic expression of ACACA in Duroc pigs (Suppl. Table S3). This may suggest a synergistic process resulting in preference of this particular allele. For ADIPOR1, FASN, and ME1, there were no significant deviations of allelic transcript levels between cDNA and gDNA in any breed or tissue (data not shown).

We then focused on searching for epigenetic regulatory elements located in 5′-flanking regions of ACACA and SCD where CGP islands were annotated according to the reference
Sscrofa 11.1 assembly. For analysis of CpG methylation level, we selected samples showing most extreme allelic imbalance (mean log_{10}-transformed allelic transcript ratio for ACACA in visceral fat: 0.21 ± 0.03; and for SCD in visceral fat: 0.23 ± 0.06 and in l. dorsi: 0.28 ± 0.05) versus control groups showing similar expression of both alleles (mean log_{10}-transformed allelic transcript ratios: 0.02 ± 0.01, 0.04 ± 0.02, and 0.06 ± 0.04, respectively). The study investigated methylation in two CpG islands (CGi) in the ACACA promoter region in DNA isolated from visceral fat: CGi1 (five CpG sites) and
CGi2 (nine CpG sites). We also investigated a CGI located in the SCD promoter region, where six CpG sites were analyzed in visceral fat and l. dorsi muscle. The mean percentage of methylated cytosines was low, 3–8% for CGI1 and 6–12% for CGI2 in ACACA, and 3–10% for the CGI in SCD in visceral fat and l. dorsi muscle (Suppl. Fig. S2) which was as expected for actively transcribed genes (Meier and Recillas-Targa 2017). The comparison of mean CpG methylation level between groups revealed no significant effects on allelic imbalance of ACACA and SCD.

Finally, to study possible association of candidate SNPs in the TNF 5′-regulatory region (rs328373700, c.-791C>T) and in 3′UTR of ACACA (rs321308225, c.*195C>A) with mRNA expression in two fat deposits and l. dorsi muscle, allelic transcript ratios from animals carrying different genotypes were compared. This strategy reduces any confounding effects of trans-regulatory and environmental factors on mRNA expression because transcript abundance is compared within the same individual, not between individuals (Forton et al., 2007). The candidate SNPs selected for this analysis were previously reported as associated with fat deposition and carcass traits (Stachowiak et al. 2013; Szydłowski et al. 2011). Due to the genotype distribution in our groups heterozygous for rSNPs (Suppl. Table S4), we could perform this study to test the regulatory effect of rs321308225 (c.*195C>A) SNP in PL breed, only. Although animals carrying CA genotype showed more imbalanced ACACA allelic expression than CC homozygotes in all tissues analyzed, the differences were not statistically significant (Suppl. Table S5). We previously reported that ACACA shows a distinct expression pattern in subcutaneous fat and l. dorsi muscle of PL pigs (Stachowiak et al. 2013), and the positioning of chromosome territory carrying ACACA has been correlated with its transcriptional activity in porcine adipocytes (Kociuçka et al. 2012) but the mechanism of its tissue-specific regulation remains unknown.

This is the first study that shows differential allelic expression of ACACA, LEP, SCD, and TNF in fat deposits and skeletal muscle of postnatal pigs. The recently reported allelic imbalance of two genes, PPARG and PPARGC1A, out of four analyzed (Stachowiak et al. 2018) suggests that this phenomenon may be widespread among genes involved in regulating lipid metabolism in pigs. Interestingly, SCD was previously shown as imbalanced in porcine prenatal skeletal muscle, whereas LEP but not FASN, SCD, and TNF displayed differential allelic expression in bovine liver, pituitary, and kidney (Olbrromski et al. 2013; Yang et al. 2016). The strategy to search for functional regulatory elements based on analysis of allelic expression has successfully revealed cis-regulatory variants governing expression of IL13 in human lymphoblastoid B cell lines and ADRB2 in porcine l. dorsi muscle (Forton et al. 2007; Murani et al. 2013). Such an approach should also be adopted to investigate the molecular basis of extensive allele-specific expression observed in pig fat tissue (Schachtschneider et al. 2015) or a skeletal muscle.

In conclusion, of seven genes analyzed, ACACA, LEP, SCD, and TNF, exhibited significant allelic imbalance in fat deposits and skeletal muscle and these genes are interesting candidates for investigation of cis-regulatory factors as potential molecular targets to modulate porcine fatness traits. Such a study should also include other elements than the linear DNA sequence, for example, cis-regulatory chromatin modifications or three-dimensional architecture of chromatin domains in the interphase nucleus.

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Author’s contributions MS designed the study, performed experiments, analyzed data, and wrote the manuscript; KF performed experiments and revised the manuscript.

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| Gene | Breed | Subcutaneous fat | Visceral fat | L. dorsi muscle | gDNA |
|------|-------|------------------|--------------|----------------|------|
| ACACA | PLW | 0.094 ± 0.054*** | 0.091 ± 0.059** | 0.093 ± 0.053*** | 0.026 ± 0.025 |
|      | PL | 0.094 ± 0.062*** | 0.104 ± 0.076*** | 0.074 ± 0.055** | 0.032 ± 0.018 |
|      | Duroc | 0.101 ± 0.057* | 0.122 ± 0.053** | 0.094 ± 0.047* | 0.039 ± 0.039 |
|      | Pietrain | 0.113 ± 0.047*** | 0.118 ± 0.062*** | 0.101 ± 0.068* | 0.041 ± 0.030 |
| LEP | PLW | 0.085 ± 0.071* | 0.076 ± 0.052* | 0.105 ± 0.099* | 0.036 ± 0.035 |
|      | Duroc | 0.083 ± 0.048*** | 0.079 ± 0.078* | 0.106 ± 0.057*** | 0.026 ± 0.021 |
| SCD | PLW | 0.106 ± 0.044*** | 0.127 ± 0.109* | 0.180 ± 0.116** | 0.037 ± 0.032 |
|      | Duroc | 0.093 ± 0.052*** | 0.094 ± 0.053** | 0.174 ± 0.120** | 0.033 ± 0.031 |
| TNF | PLW | 0.120 ± 0.107** | 0.104 ± 0.059*** | 0.126 ± 0.139* | 0.026 ± 0.021 |
|      | PL | 0.140 ± 0.113*** | 0.118 ± 0.144* | 0.168 ± 0.132** | 0.028 ± 0.026 |

Data were calculated after neutralizing bi-directional character of allelic imbalance. Significant differences between cDNA and gDNA are shown at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).
Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethics approval All animal procedures were approved by the Local Ethical Commission on Experiments on Animals at the Poznan University of Life Sciences, Poznan, Poland (no. 57/2012).

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