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Short communication

SNPs IN THE PORCINE *PPARGC1A* GENE: INTERBREED DIFFERENCES AND THEIR PHENOTYPIC EFFECTS

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Abstract: Due to its function, the peroxisome proliferative activated receptor-γ, coactivator-1α (*PPARGC1A*) gene is a candidate in the search for genes that may affect production traits in the pig. The purpose of this study was to screen for new SNPs in exon 8 of the porcine *PPARGC1A* gene and to test their possible association with production traits. Altogether 736 pigs representing five breeds (Polish Landrace, n=242; Polish Large White, n=192; Hampshire, n=27; Duroc, n=21; Pietrain, n=12) and synthetic line 990 (n=242) were scanned via SSCP assay. Four SNPs were found; two new ones: C/G (His338Gln) and G/A (Thr359Thr), and two previously reported ones: C/A (Arg369Arg) and T/A (Cys430Ser). The missense T/A and C/G SNPs demonstrated pronounced interbreed variability in terms of allele frequencies, including the exclusive presence of the C/G substitution in the Hampshire breed. The tested SNPs occurred in five putative haplotypes, and their frequency also differed

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Abbreviations used: AF – abdominal fat weight; BF – backfat thickness; FC – feed conversion ratio; *FOXO1* – forkhead box O1; *FXR* – farnesoid X receptor; *GR* – glucocorticoid receptor; *HNF4α* – hepatic nuclear factor 4α; IMF – intramuscular fat content; LD – *longissimus dorsi*; *LXRα* – liver X receptor-*α*; *MEF2* – myocyte enhancer factor 2; *NRF*-1 – nuclear respiratory factor 1; *NRF*-2 – nuclear respiratory factor 2; PCR – polymerase chain reaction; PL – Polish Landrace; PLW – Polish Large White; *PPARα* – peroxisome proliferative activated receptor-α; *PPARβ/δ* – peroxisome proliferative activated receptor-β/δ; *PPARGC1A* – peroxisome proliferative activated receptor-γ, coactivator-1α; QTL – quantitative trait loci; RFLP – restriction fragment length polymorphism; *RYR1* – ryanodine receptor 1; SNP – single nucleotide polymorphism; SSC8 – *Sus scrofa* chromosome 8; SSCP – single strand conformation polymorphism

substantially between breeds. The association of the SNPs with production traits was tested for G/A (Thr359Thr), C/A (Arg369Arg) and T/A (Cys430Ser) substitutions in Polish Large White, Polish Landrace and line 990. The analysis revealed only breed-specific associations. The T/A (Cys430Ser) SNP was related to the feed conversion ratio in the Polish Large White (P=0.02), and the silent G/A and C/A substitutions were respectively associated with abdominal fat in line 990 and backfat thickness in Polish Landrace (P=0.04). The combined effects of the substitutions were estimated as haplotype effects. Three significant contrasts between haplotypes were calculated, but the observed associations were again only breed-specific.

Key Words: Pig, *PPARGC1A* gene, Quantitative trait loci, SNP

INTRODUCTION

Peroxisome proliferative activated receptor-γ, coactivator-1α (PPARGC1A) is a highly versatile coactivator of multiple ligand-dependent transcription factors, and is involved in the regulation of energy homeostasis, adaptive thermogenesis and muscle fiber type formation [1]. Because of its location, function and genetic variability, the *PPARGC1A* gene has recently emerged as a candidate gene for meat quality and fatness traits in livestock.

Of almost 800 QTLs reported to date in the pig, at least ten affecting growth rate and body composition traits have been assigned to the region between the Sw905 and Sw933 markers of the porcine chromosome 8 (SSC8), where the *PPARGC1A* gene was mapped [2-6]. A T/A substitution in exon 8 of the *PPARGC1* gene resulting in a Cys430Ser change was suggested to have an impact on fat deposition when Chinese and Western pig breeds were compared [7]. However, the haplotype study performed by Jacobs *et al.* [8] showed that the T/A substitution had no effect on production traits in the Meishan cross resource population, but another SNP – a missense mutation A/C (Pro615Thr) in exon 9 – demonstrated such an effect. The aim of this study was to screen exon 8 of the *PPARGC1A* gene for new SNPs encoding domains interacting with multiple transcription factors, and to analyze their possible phenotypic effect on production traits in Polish pig breeds.

MATERIALS AND METHODS

A total of 714 gilts, representing synthetic line 990 (n=242), and the Polish Landrace (PL; n=242), Polish Large White (PLW; n=192), Duroc (n=21), Pietrain (n=12) and Hampshire (n=5) breeds were genotyped. Synthetic line 990 was derived by crossing Polish Large White, Duroc, Hampshire and three lines of Landrace pigs. The gilts were fed *ad libitum* up to 100 kg body weight and slaughtered and dissected at the Pig Testing Station (Pawlowice, Poland). Nine fatness traits were recorded: abdominal fat weight (AF), intramuscular fat content (IMF), and seven measurements of backfat thickness (BF) – over the

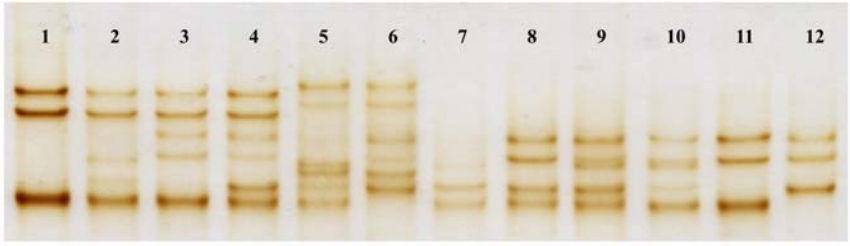
shoulder, at the last rib, at the *sacrum* (points I, II and III), over the *longissimus dorsi* muscle (LD) and over the side of LD. The IMF was measured using the SOXTEC AVANTI 2050 extraction system (Foss Tecator, Sweden). The four other analyzed traits were: average daily gain, feed conversion ratio (FC), lean meat content, and loin eye area. Additionally, 22 Hampshire pigs kept at the Animal Breeding Centre (Chodeczek, Poland) underwent molecular analysis. However, their biometric and performance data was not available.

The genomic DNA from the 714 pigs was extracted from blood using a Perfect gDNA Blood Mini Isolation Kit (A&A Biotechnology, Gdansk, Poland). The genomic DNA originating from the 22 Hampshire pigs was extracted from hair follicles. Six hair follicles were cut, placed in 200 mM NaOH and heated according to the protocol: 75ºC/1 min, 80ºC/2 min, 90ºC/1 min and 97ºC/10 min. After heating, 200 mM HCl in 100 µM Tris-HCl (pH 8.5) were added. The samples were then mixed and stored at -20ºC. PCR primers (5'-ccacccaccactcctcctcataaa-3' and 5'-ttgtctgcttcgtcgtcaaaaaca-3') were designed based on the porcine *PPARGC1A* mRNA sequence (GenBank, AY346131S1). Each 20 µl PCR reaction contained: 100 ng DNA, 0.4 µM of each primer, 0.125 mM of each dNTP and 1 U *Taq* polymerase (Nowazym, Poland). The amplification conditions were 5 min at 94ºC, 35 cycles of 30 s at 94ºC, 30 s at 61ºC and 1 min at 72ºC, and a 5-min final extension at 72ºC. The 554-bp amplified fragment was subjected to SSCP analysis. Electrophoresis was performed on a 9% polyacrylamide gel (acrylamide: bisacrylamide 49:1, 1xTBE, 5% glycerol). Four microliters of PCR product were mixed with 20 µl buffer (80% formamide, 6% glycerol, 0.25% bromophenol blue, 0.25% xylenecyanol), denatured for 5 min at 97ºC, and rapidly chilled on ice. Following 26 h electrophoresis at 200 V at 10ºC, the gel was silver stained. One PCR product from each SSCP variant was sequenced on both strands at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Warsaw, Poland). The PCR-RFLP assay was performed with *Tsp*RI (NEB) and *Btg*I (NEB) endonucleases according to the manufacturer's recommendations, and separated on a 1.8% agarose gel. Haplotypes were predicted excluding double heterozygotes. The association between the 13 production traits and 3 SNPs was tested separately within PLW, PL and line 990. The statistical model included the tested genotype at the polymorphic site as a fixed effect and the sire as random. Other effects were introduced to the model for a particular trait if the corresponding F value reached 2. The effects and the covariables considered were genotype at the *RYR1* locus, carcass weight and age at slaughter for carcass traits, and average BF (from 7 measurements) for IMF. Additionally, the missense T/A mutation (Cys430Ser) was tested in the across-breed analysis including breed as a fixed effect. The combined effects of substitutions were estimated as haplotype substitution effects. Three haplotype effects were estimated simultaneously as the regression coefficients, constraining their sum to zero.

RESULTS

All the animals involved in the study were screened for polymorphism in exon 8 of the *PPARGC1A* gene using the PCR-SSCP technique. Twelve different SSCP patterns were observed (Fig. 1). DNA samples representing each SSCP pattern were subsequently sequenced and four SNPs, including both missense and silent substitutions were found. Two of them were previously described. The DNA sequence together with the identified polymorphisms was deposited in the GenBank database (DQ449507).

The known T/A missense mutation resulting in the Cys430Ser substitution in the coactivator protein was already reported by Kunej *et al.* [7]. Since the annotation of SNPs in exon 8 of the *PPARGC1A* gene is not consistent between studies, we decided to standardize it by numbering based on the position in the coding sequence starting from the ATG codon. The position of this SNP in the coding sequence was 1288, which corresponded to position 1378 (GenBank, AY346131S1, [7]) and 678 (GenBank, AY484500, [8]) indicated in previous studies. The PCR-SSCP assay was sufficient to distinguish three genotypes at position 1288; therefore, the PCR-RFLP analysis with the use of *TspR*I endonuclease was abandoned and further genotyping was performed using only the PCR-SSCP technique. The observed allele frequencies differed substantially between breeds (Tab. 1). In line 990, and Duroc and Polish Landrace (PL), allele A was predominant, whereas in the Polish Large White (PLW), Hampshire and Pietrain breeds the T allele was more frequent. There was no deviation from the Hardy-Weinberg equilibrium in the tested groups. The overall T allele frequency in the 736 studied pigs was 0.412 (data not shown).



SNP	1	2	3	4	5	6	7	8	9	10	11	12
1014	CC	CC	CC	CC	CG	CG	CC	CC	CC	CC	CC	CC
1077	AA	AG	AG	AG	GG	GG	GG	GG	GG	GG	GG	GG
1105	CC	AC	CC	CC	AC	CC	AA	CC	AC	AC	CC	CC
1288	TT	TT	TT	AT	AT	AA	TT	AT	AT	TT	TT	AA
Haplotype genotype	CACT / CACT	CGAT / CACT	CACT / CGCT	CACT / CGCA	CGAT / GGCA	CGCA / GGCA	CGAT / CGAT	CGCA / CGCT	CGCA / CGAT	CGCA / CGAT	CGCT / CGCT	CGCA / CGCA

Fig. 1. Twelve different SSCP patterns and the corresponding genotypes and putative haplotypes at four positions: 1014 (His338Gln), 1077 (Thr359Thr), 1105 (Arg369Arg) and 1288 (Cys430Ser) of the *PPARGC1A* coding sequence (exon 8).

The other missense mutation identified in the analyzed fragment of exon 8 is a newly identified C/G substitution at position 1014 of the coding sequence. As a result of this SNP, a histidine at position 338 of the PPARGC1A protein is replaced by glutamine. This genotype can be detected by restriction with *Btg*I endonuclease. In the case of the C allele, the 554-bp PCR product is cleaved into two bands (422 and 132 bp), whereas in the presence of the G allele, the DNA fragment remains undigested. The PCR-SSCP screening of 736 animals revealed that the G allele appeared exclusively in the Hampshire breed (Tab. 1). This was subsequently confirmed by *Btg*I PCR-RFLP genotyping of all the Hampshire pigs and 33 randomly selected, unrelated gilts, representing other breeds. Allele G was distributed in the Hampshire breed with a frequency of 0.111, but the GG genotype was not found due to the small size of the sample.

The silent substitutions identified in the studied fragment included a new G/A transition at position 1077 (Thr359Thr) and a previously described C/A (Arg369Arg) transversion at position 1105 (GenBank, AY346131S1). The distribution of the alleles and genotypes across breeds is presented in Tab. 1. Both SNPs were distributed unevenly in the tested breeds with the G and C alleles being predominant at positions 1077 and 1105, respectively. The overall allele frequency in the studied pigs was 0.961 for the G allele and 0.853 for the C allele (data not shown).

The analyzed SNPs occurred in five putative haplotypes: $C_{1014}G_{1077}C_{1105}A_{1288}$, $C_{1014}G_{1077}C_{1105}T_{1288}$, $C_{1014}G_{1077}A_{1105}T_{1288}$, $C_{1014}A_{1077}C_{1105}T_{1288}$ and $G_{1014}G_{1077}C_{1105}A_{1288}$ (Fig. 1). Their distribution differed between breeds, and some haplotypes appeared to be breed-specific (Tab. 1). The $C_{1014}G_{1077}C_{1105}A_{1288}$ haplotype was predominant in the PL, Duroc and Pietrain breeds and line 990, while the $C_{1014}G_{1077}C_{1105}T_{1288}$ haplotype was more frequent in the PLW gilts. The $C_{1014}A_{1077}C_{1105}T_{1288}$ haplotype was found only in line 990, Duroc and Pietrain pigs. The $G_{1014}G_{1077}C_{1105}A_{1288}$ haplotype was specific to the Hampshire breed.

Due to the sample size, a statistical analysis of the effect of the studied SNPs could only be performed for PLW, PL and line 990. The data gave no strong evidence for an association between the tested SNPs and the production traits measured. Due to the low degree of the G/A polymorphism at the 1077 site (Thr359Thr), the only comparison made was that between the AG and GG genotype within line 990. The smallest P value of 0.04 was calculated for AF. The least square means were 0.47 ± 0.028 kg and 0.53 ± 0.014 kg for AG and GG, respectively. The test for an association between the C/A SNP at the 1105 position (Arg369Arg) and the production traits was based on a comparison between AC and CC gilts within PLW and PL. The most significant result was obtained for the BF over back within PL (P = 0.04, AC: 13.0 ± 0.6 mm, CC: 11.7 ± 0.14 mm). For the missense T/A mutation at position 1288, all three genotypes were included in the test except for the TT gilts of line 990. Breed specific analyses showed a significant result (on individual test error rate) for FC, but only

Tab. 1. The genotype, allele and haplotype frequency of porcine *PPARGC1A* in different breeds.

SNP	Genotype Allele Haplotype[a]	Breed[b]					
		PLW (n=192)	PL (n=242)	Line 990 (n=242)	Hampshire (n=27)	Duroc (n=21)	Pietrain (n=12)
1014	CC	1.000	1.000	1.000	0.778	1.000	1.000
(His338Gln)	CG	-	-	-	0.222	-	-
	C	1.000	1.000	1.000	0.889	1.000	1.000
	G	-	-	-	0.111	-	-
1077	AA	-	-	0.004	-	-	-
(Thr359Thr)	AG	0.005	0.008	0.157	0.259	0.190	0.250
	GG	0.995	0.992	0.839	0.741	0.810	0.750
	A	0.003	0.004	0.083	0.130	0.095	0.125
	G	0.997	0.996	0.917	0.870	0.905	0.875
1105	AA	0.021	0.041	-	0.074	-	-
(Arg369Arg)	AC	0.156	0.525	0.033	0.556	0.048	0.250
	CC	0.823	0.434	0.967	0.370	0.952	0.750
	A	0.099	0.304	0.017	0.352	0.024	0.125
	C	0.901	0.696	0.983	0.648	0.976	0.875
1288	AA	0.109	0.289	0.665	0.148	0.524	0.333
(Cys430Ser)	AT	0.516	0.521	0.306	0.556	0.286	0.250
	TT	0.375	0.190	0.029	0.296	0.190	0.417
	A	0.367	0.550	0.818	0.426	0.667	0.458
	T	0.633	0.450	0.182	0.574	0.333	0.542
Haplotype	CGCA	0.356	0.586	0.882	0.333	0.722	0.435
	CGCT	0.576	0.240	0.099	0.208	0.250	0.391
	CGAT	0.068	0.174	0.002	0.333	-	0.087
	CACT	-	-	0.017	-	0.028	0.087
	GGCA	-	-	-	0.126	-	-

[a]Haplotype at nucleotide positions 1014, 1077, 1105 and 1288 of the *PPARGC1A* coding sequence, [b]PLW: Polish Large White, PL: Polish Landrace

in PLW (P=0.02, AA: 2.51 ± 0.097 kg, AT: 2.72 ± 0.063 kg, TT: 2.73 ± 0.061 kg). However, the across-breed analysis yielded no significant results. The haplotype substitution effects were calculated for each breed and across breeds. Three significant results were observed for the contrasts between haplotypes: for AF in PL between CGCA and CGCT (P=0.01), and between CGAT and CGCT (P=0.04, CGAT: 31 ± 20.1 g, CGCA: 19 ± 12.2 g, CGCT: -50 ± 20.9 g), and for BF over shoulder in PLW between CGCA and CGCT (P = 0.04, CGCA: -3.1 ± 4.1 mm, CGCT: 9.2 ± 5.2 mm). No significant results were found in the across-breed analysis.

DISCUSSION

The peroxisome proliferative activated receptor- γ, coactivator-1α is one of the most potent and highly regulated coactivators which integrates signaling pathways at the level of gene transcription in mammals. The target genes regulated by PPARGC1A are involved in fatty acid oxidation (*PPARα*, *PPARβ/δ*), mitochondrial biogenesis (*NRF-1*, *NRF-2*) lipoprotein secretion (*LXRα* and *β*), triglyceride metabolism (*FXR*), gluconeogenesis (*GR*, *FOXO1* and *HNF4α*) and slow-twitch muscle fiber specification (*MEF2*) [1]. Since the PPARGC1A protein is important for energy metabolism regulation, its potential effect on performance and body composition traits in livestock has been extensively investigated. It is conserved among species and contains a number of domains and motifs interacting with transcriptional factors. The amino acid substitutions localized within these functional regions may affect the protein's activity.

In this study, we scanned exon 8 of the porcine *PPARGC1A* gene and detected four SNPs. Of these, two were missense substitutions that could exert a potential phenotypic effect. A previously described T/A mutation results in the replacement of cysteine by serine in position 430 of the protein, which is suggested to be involved in the interaction with MEF2-C, a transcription factor, regulating glucose transport in muscle [9]. This T/A (Cys430Ser) polymorphism was previously implied to be functional in the study of Kunej *et al.* [7], where the mean frequency of the T allele differed significantly between Chinese (n=101) and Western (n=138) pig breeds (0.93 and 0.46, respectively). In our investigation, the allele T frequency differed substantially between breeds as well. The overall T allele frequency (0.412) was close to that reported for Western breeds by Kunej *et al.* [7]. The T allele distribution was within the range of 0.182 to 0.633 for line 990 and the PLW breed, respectively, while in the study of Kunej *et al.* [7], it ranged from 0.29 to 0.67 for German and Swedish Landrace, respectively. Such diversity may prove that this SNP is not associated with production traits, since no selection pressure was applied to eliminate the undesired allele. On the other hand, it may underlie the interbreed variability, reflecting breed-specific performance of the production traits.

The new C/G SNP resulting in His338Gln substitution was detected exclusively in Hampshire pigs. Both histidine and glutamine are polar amino acids and are often involved in protein active or binding sites. However, the unique property of histidine is that it can easily change the charge of the side chain from neutral to positive. This flexibility makes it the ideal residue for protein functional centers catalyzing the creation and cleavage of bonds. Exchanges of histidine for any other amino acid are relatively rare and may affect the protein activity [10]. This substitution is localized in the region of the PPARGC1A protein found to be functional in rodents. The domain between amino acids 180 and 403 interacts with the NRF-1 transcription factor [11]. The PPARγ-binding domain was designated in the central region of the protein, between amino acids 292 and 403

[11, 12]. Also, the putative negative regulatory domain was shown to be located between amino acids 170 and 350 [13]. Therefore, the C/G substitution at position 1014 may potentially exert a phenotypic effect. Unfortunately, due to the very small sample of available Hampshire pigs, a statistical analysis of the association was not possible.

The association study gave limited evidence that the studied polymorphisms may be associated with variability of production traits. However, although some small P values were calculated, the results were considered insignificant when adjusting for multiple tests (Bonferroni correction). The power of the performed association study was limited by the distribution of the polymorphisms. Only for the missense T/A (Cys430Ser) mutation were the alternative homozygotes available for comparison and the sample sizes large enough to detect a functional SNP of a medium effect. Our results suggest that the Cys430Ser polymorphism may have some impact on the feed conversion ratio in the Polish Large White breed. The standardized linkage disequilibrium (D') between Cys430Ser and Arg369Arg could be calculated for two breeds only, and reached 66% in PLW and 62% in PL. This suggests that within the *PPARGC1A* gene, various SNPs may appear independently. Our findings do not exclude the possibility that some functional mutations exist within the gene. As the missense C/G (His338Gln) polymorphism at position 1014 was found exclusively in the Hampshire breed, it is reasonable to recommend further studies on its potential functionality. The interbreed difference of the allele frequency at position 1014 may be interpreted as a result of the genetic drift or linkage disequilibrium or selection or both.

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