

SNP detection in the porcine *PPARGC1A* promoter region and 3'UTR, and an association analysis in a Landrace-Duroc-Yorkshire population

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ABSTRACT: Meat quality is of increasing economic importance to the pork industry today, which is in contrast with the more traditional focus of pig selection for lean growth. Meat quality is however determined by many factors with a complex mutual relationship. In this regard, *PPARGC1A* is a very interesting candidate gene because it not only plays a crucial role in energy and fat metabolism but also has an important influence on the muscle fibre type composition. However, only little is known about the regulation of expression of this gene in the pig and its usefulness in pig selection. In order to get a better understanding of the regulation of *PPARGC1A* expression, 1 898 base pairs (bp) from the promoter region and the complete 3'UTR (3 826 bp) were sequenced and screened for mutations in 7 diverse pig breeds. Respectively 5 and 6 new mutations were discovered in these regions, of which several were in complete linkage disequilibrium with each other. None of the detected SNPs appeared to be located in any conserved part of the sequence when comparing different species. In an association analysis with intramuscular fat percentage, leaf fat weight or last rib backfat depth carried out in a Landrace-Duroc-Yorkshire commercial research population ($n = 960$), no associations were detected for the new SNPs from this study or for 2 previously described SNPs in exon 8 and 9. The results from this study provide essential information on the sequence of the promoter region and 3'UTR of porcine *PPARGC1A*, necessary for unravelling the complex regulation of expression and functioning of this gene in the pig. Although no association with meat quality and fat deposition parameters was found for the newly discovered SNPs in the regulatory regions, these need to be used in future studies to (further) assess their usefulness as new selection criteria for improving meat quality while maintaining the leanness of the carcass.

Keywords: association analysis; fat deposition; meat quality; pig; *PPARGC1A*; promoter region; SNP; 3'UTR

Meat quality and carcass composition characteristics are of great importance to the pork industry. Whereas the more classical criteria of pig selection were focussed on lean growth, feed conversion and high growth rate, the improvement of meat quality is nowadays of increasing interest due to the strong

influence of consumer demands (Karlsson et al., 1999; Yu et al., 2006; Davoli and Braglia, 2007). Among others, backfat and intramuscular fat (IMF) content are important parameters of meat quality and carcass composition, which are strongly influenced by the genetic composition of the animal

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(Cameron, 1990; Rohrer and Keele, 1998). One of the main difficulties is, however, selecting towards an improved meat quality while maintaining a lean carcass (Fiedler et al., 2003; Maltin et al., 2003).

In order to get a better understanding of the multiple factors determining meat quality, fat deposition and their complex relationship, it is important to identify the genes involved. In this regard, the peroxisome proliferator-activated receptor γ coactivator 1 α (*PPARGC1A*) is a very interesting candidate gene affecting meat quality. A quantitative trait locus for leaf fat weight was located in the chromosomal region where the porcine *PPARGC1A* was mapped (Jacobs et al., 2006) and other candidate genes for meat quality (e.g. *SLC2A4*, previously known as *GLUT4*) are controlled by *PPARGC1A* (Michael et al., 2001). *PPARGC1A* is a coactivator of multiple nuclear hormone receptors and other transcription factors, and in this way exerts an influence on many genes, mainly involved in energy and fat metabolism (Puigserver et al., 1998; Wu et al., 1999). This cold- and exercise-inducible coactivator is an essential regulator of adipogenesis, adipocyte differentiation, mitochondrial biogenesis/respiration and adaptive thermogenesis. It is highly expressed in tissues with a high energy demand such as muscle, brown fat and brain (Larrouy et al., 1999; Spiegelman et al., 2000; Oberkofler et al., 2002; Medina-Gomez et al., 2007). Furthermore, *PPARGC1A* increases the amount of oxidative muscle fibres and is higher expressed in this tissue, which indicates that *PPARGC1A* is important in the muscle fibre type differentiation (Lin et al., 2002; Zhao et al., 2004; Mortensen et al., 2006). In general, more oxidative and less glycolytic fibres have a positive impact on meat quality, but this also depends on the location of the muscle and the breed (Klont et al., 1998; Chang et al., 2003). In contrast, classical pig selection for lean growth often decreased meat quality, because it resulted in the presence of less IMF (Yu et al., 2006) and of fewer oxidative and more glycolytic fibres (Brocks et al., 2000).

Several single nucleotide polymorphisms (SNPs) have been identified in both humans and pigs in the coding region of *PPARGC1A*, which respectively could be associated with mainly obesity/type II diabetes and fat characteristics (Ek et al., 2001; Kunej et al., 2005; Jacobs et al., 2006; Vimalleswaran et al., 2006; Stachowiak et al., 2007). Polymorphisms have also been found in the bovine promoter region and the human and bovine 3' untranslated region

(3'UTR; Esterbauer et al., 2002; Weikard et al., 2005). Furthermore, it has been shown that the porcine mRNA expression of *PPARGC1A* differs between the economically important tissues backfat and *longissimus dorsi muscle*, and that the expression also varies within this muscle (Erkens et al., 2006). This last observation indicates that *PPARGC1A* expression depends on the location within the same muscle, possibly related to the muscle fibre type composition and influencing meat quality (Klont et al., 1998). However, only very little is known about the regulation of *PPARGC1A* expression and its usefulness in pig selection. We therefore sequenced the promoter region and 3'UTR in the pig, after which both regions were screened for mutations in 7 diverse pig breeds in an attempt to identify polymorphisms with a possible influence on *PPARGC1A* expression, meat quality and fat deposition. These newly discovered mutations and the ones identified by our group in a previous study (Jacobs et al., 2006) were also used in an association analysis with a Landrace-Duroc-Yorkshire population, to unravel possible relationships with intramuscular fat percentage, leaf fat weight and last rib backfat depth, which are important meat quality and carcass composition traits possibly influenced by *PPARGC1A*.

MATERIAL AND METHODS

Samples and DNA preparation

Genomic DNA from 7 pig breeds (5 Piétrain, 14 Large White, 8 Landrace, 9 Duroc, 5 Black Pied Prestice, 5 Meishan and 5 wild boars) was isolated with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) for muscle samples, from blood using proteinase K, or was available as ready-to-use genomic DNA (provided by Prof. Antonín Stratil).

Primers and sequencing

NCBI and Ensembl databases were searched for sequences of the promoter region and 3'UTR of *PPARGC1A* from pig, human, mouse, rat and cow to identify (preferably conserved) regions which could be used for primer design with Primer3 (Rozen and Skaletsky, 2000). Mfold (Zuker, 2003) and Blast (Altschul et al., 1990) were used to check for pos-

Table 1. Primers used for sequencing and mutation screening

Primer name	Primer sequence (5'→3')	Amplicon length	Ta	Accession number
PGC1A-1F	TGCCCCGTTCTCTGCTTT	563 bp	64°C	EU088376 (promoter region)
PGC1A-1R	AACCAGCCCCTTACTGAGAGTG			
PGC1A-2F	CAGGGCACTAGGGTTGGA	464 bp	63°C	
PGC1A-2R	TTCTAATTGGGGGAAGTGTTTG			
PGC1A-3F	TCTTTGTGGGGAGTGCAGAGT	1 017 bp	62°C	
PGC1A-3R	CTTTGCCAGATCAGCTTAGTTTC			
PGC1A-4F	CAGCAAAGGATACCTCTTACCA	1 087 bp	64°C	
PGC1A-4R	AGAAAGAACCGCTGAACAAG			
PGC1A-5F	CCACAAGCCACCTAAAATG	394 bp	62°C	
PGC1A-5R	AGAAAGAACCGCTGAACAAG			
PGC1A-6F	TCAATGGTCAAGCGTGCAG	1 055 bp	58°C	
PGC1A-6R	CCCAAATGAAGCACTTACTACTCC			
PGC1A-7F	GAAGCCAGTATTTCTTTCCCTTG	1 302 bp	58°C	EU088376 (3'UTR)
PGC1A-7R	GCACAGCTTACATTTTGATGG			
PGC1A-8F	CCCTGGATTGTTGACATGAG	915 bp	58°C	
PGC1A-8R	ATGTAGAATTGGCAGGTGGA			
PGC1A-9F	TGTGAAGTCTGATTTGATGG	297 bp	61°C	
PGC1A-9R	AACATGCCAGGAGTTTGGT			
PGC1A-10F	ATCCTTCCAGTGCAACAAAA	489 bp	60°C	
PGC1A-10R	TCATACAATGAATAAAACCACAACA			

Ta = annealing temperature

sible secondary structures and primer specificity, respectively (Table 1). All PCRs were optimized with FastStart Taq DNA Polymerase (Roche) and included a no-template control. Sequencing of the amplicons was conducted on an Applied Biosystems 3730xl DNA Analyser with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's protocol.

Sequence analysis and mutation detection

By comparing the sequences of the PCR fragments produced by all primer pairs from the 51 pigs with MultAlin (Corpet, 1988), a consensus sequence of the promoter region and 3'UTR was assembled and at the same time both regions were screened for mutations. The location of the detected mutations in the porcine sequence was compared to the

known human, mouse, rat and bovine sequences (EMBOSS) to assess whether they are located in any conserved regions of the gene. The presence of any repeat regions was verified using the program RepeatMasker (Smit et al., 2004). For the promoter region, MatInspector (Cartharius et al., 2005) was used to determine if the detected SNPs are located in putative promoter elements.

Association analysis

An association analysis using 3 fat deposition parameters was conducted with the newly discovered SNPs listed in Table 2 and with 2 previously described SNPs in exon 8 and 9 (SNP12 (AY484500:g.678T>A) and SNP13 (AY484500:g.1747C>A), respectively; Jacobs et al., 2006). The commercial research population was developed by mating

Table 2. Porcine *PPARGC1A* SNPs, allele frequencies and an association analysis in a Landrace-Duroc-Yorkshire population

	Mutation ^c	Allele frequency in Landrace-Duroc-Yorkshire ^d	IMF ^f	Backfat ^f	Leaf fat ^f	Location
SNP1	g.237C>G	ND	–	–	–	
SNP2 ^a	g.471G>A g.479G>A	100.00% (G)	–	–	–	promoter region
SNP3	g.654A>G	ND	–	–	–	
SNP4	g.698C>G	93.02% (C) ^e	0.6482 ^g	0.3786 ^g	0.1495 ^g	
SNP5	g.1487G>A	93.02% (G) ^e	0.6482 ^g	0.3786 ^g	0.1495 ^g	
SNP6 ^b	g.2885C>T g.2886T>C g.2888C>T g.2889T>C g.2895C>T	ND	–	–	–	
SNP7	g.4844T>C	ND	–	–	–	3'UTR
SNP8	g.5018C>T	48.96% (C)	0.3442	0.9655	0.9360	
SNP9	g.5452G>T	96.98% (G)	–	–	–	
SNP10	g.5460C>T	68.02% (C)	0.1297	0.8109	0.4775	
SNP11	g.5842T>G	ND	–	–	–	
SNP12	g.678T>A	45.00% (A)	0.5342	0.5958	0.5342	exon 8
SNP13	g.1747C>A	91.04% (C)	0.9032 ^g	0.2213 ^g	0.4940 ^g	exon 9

^aSNP2 consists of 2 polymorphisms in complete linkage disequilibrium

^bSNP6 consists of 5 polymorphisms in complete linkage disequilibrium

^cSNP location is based on GenBank accession number EU088376 for SNP1-SNP11, and on AY484500 for SNP12-SNP13

^dND, no data available

^eSNP4 and SNP5 were in complete linkage disequilibrium in the LDY research population

^f*P*-values of the association analyses for SNP markers with IMF percentage, last rib backfat depth and leaf fat weight

^gdue to low minor allele frequency, these results should be evaluated with caution

Yorkshire-Landrace composite females to either Duroc or Landrace boars selected from the industry. Twelve boars of each breed were selected and approximately 10 barrows per boar were slaughtered at the USMARC abattoir and phenotyped during this first cross generation (TC1; $n = 227$). The Landrace-sired pigs were crossed to Duroc-sired pigs to develop a Landrace-Duroc-Yorkshire composite population (LDY). Gilts in the F4 generation of LDY composite population were slaughtered at the USMARC abattoir and fat deposition data collected ($n = 450$) as well as barrows from the F5 generation ($n = 120$). The final population was developed by mating LDY females to a commercial terminal sire selected for either extreme pork quali-

ty or leanness (TC2). Barrows and gilts were slaughtered at the USMARC abattoir and fat deposition data collected ($n = 163$). A total of 960 records were available for intramuscular fat percentage (in %), 863 records for last rib backfat depth (ultrasonically measured prior to exsanguinations; in mm) and 881 records for leaf fat weight (in kg).

The analyses were as described by Kuehn et al. (2007), where GenoProb (Thallman, 2002) was used to evaluate the genotypic data and predict missing genotypes. Then regression coefficients for additive, dominance and imprinting genotypic effects were computed and used in a mixed model analysis using the MTDFREML program (Boldman et al., 1995). A complete animal model was fitted along

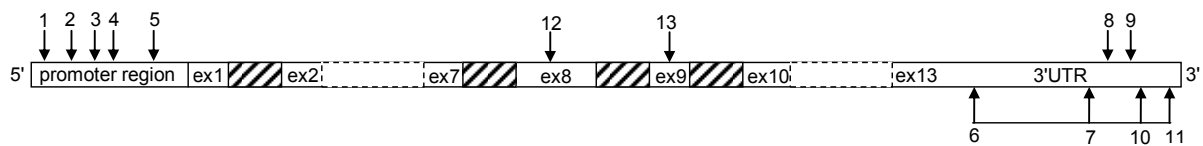


Figure 1. Schematic overview of the SNPs detected in *PPARGC1A*; numbered arrows indicate detected SNPs; connected arrows represent linked SNPs; striped box: intron

with regression coefficients for slaughter group and slaughter age. Hot carcass weight was included as a covariate for last rib backfat depth and leaf fat weight.

RESULTS

Promoter region, 3'UTR and mutation detection

In this study, 1 898 bp of the promoter region (upstream of exon 1) and the complete 3'UTR (3 826 bp) of porcine *PPARGC1A* were sequenced (EU088376). In the promoter region an A-repeat microsatellite was detected at position 1 030–1 057. Also a GA-rich (1 322–1 374) and T-rich (1 708 to 1 759) low complexity sequence were noticed. In the 3'UTR a CAA microsatellite (2 273–2 318), a TG microsatellite (2431–2485), an A-repeat microsatellite (2 630–2 663) and a low complexity AT-rich region (5187–5211) were detected.

In the promoter region and 3'UTR, 5 (SNP1–SNP5) and 6 mutations (SNP6–SNP11) were detected, respectively (Table 2; Figure 1). SNP2 actually consisted of 2 polymorphisms (EU088376: g.471G>A + g.479G>A) located 8 bp apart from each other and in complete linkage disequilibrium. SNP6 consisted of 5 polymorphisms also in complete linkage disequilibrium, within a region of 11 bp (EU088376:g.2885-2895CTTCTTT TTTC>TCTTCTTTTTT). Furthermore, SNP6, SNP7 (EU088376:g.4844T>C), SNP10 (EU088376: g.5460C>T) and SNP11 (EU088376:g.5842T>G) were in complete linkage disequilibrium with each other (alleles were CTTCTTTTTTC-T-C-T or TCTTCTTTTTT-C-T-G).

The porcine sequence of the *PPARGC1A* promoter region and 3'UTR was compared to the known human, bovine, mouse and rat sequences. The promoter region of the pig was identical for approximately 82% to the human and bovine sequence with only a few gaps, while the identity to the mouse

and rat sequence was only 65% with multiple gaps present. The first 140 bp, counting upstream from exon 1, are highly conserved between species. None of the detected SNPs are however located in highly conserved parts of the sequence or in putative promoter elements. The porcine 3'UTR (3 826 bp) shows a strong resemblance to the human and bovine sequence (91%), while the mouse and rat sequence are much shorter (just over 500 bp). Again, none of the detected SNPs are located in strongly conserved parts of the 3'UTR.

Association analysis

The results of the association analysis with intramuscular fat percentage, last rib backfat depth and leaf fat weight are summarized in Table 2. The mean percentage of IMF was 2.64%, with a standard deviation of 1.25%. The phenotypic standard deviation for last rib backfat depth was 3.31 mm and for leaf fat weight it was 0.24 kg. No significant associations with any of the 3 performance traits were detected for SNPs tested. SNP4 and SNP5 were in complete linkage disequilibrium with each other in the USMARC population, so the results are the same, but only a limited number of heterozygotes and almost no homozygotes for the minor allele were detected in the population and results for these SNPs should be evaluated with caution, just as was the case for SNP13. Therefore, due to technical difficulties or minor allele frequencies that were too low for association analysis, only SNP8, SNP10 and SNP12 could be evaluated reliably.

DISCUSSION

During years of selection for rapid lean growth in pigs, the quality of the meat clearly decreased. Today, however, meat quality is becoming increasingly important in the pig industry, but this is a complicated trait which is based on both objective

and on more subjective parameters. As explained in the introduction, *PPARGC1A* is a very interesting candidate gene for meat quality and carcass composition because it influences and regulates many processes related to fat deposition characteristics. In order to get a better understanding of the regulation of the expression of porcine *PPARGC1A* and its relation to fat deposition, in this study we sequenced and screened the promoter region and 3'UTR for mutations. The comparison of the porcine promoter region to other species revealed that several parts of this sequence are strongly conserved, particularly the region directly upstream of exon 1. Indeed, in our study no mutations could be detected in this important regulatory area. This last observation confirms the findings of Chang et al. (2006), who found several conserved promoter elements by comparing, between species, approximately the first 150 bp upstream of the 2 possible human transcription initiation sites (TIS; Esterbauer et al., 1999). Despite the fact that Esterbauer et al. (1999) described the human promoter of *PPARGC1A* as being TATA-less, when comparing sequences there does seem to be a TATA-box present in both the human and pig sequence upstream of 1 of the 2 TIS. The high resemblance of the porcine 3'UTR to the human and bovine sequence (91%) indicates that it is important for the regulation of expression of the gene. Comparison to the human gene also revealed the presence of a second polyadenylation signal (4 806–4 811), as described in humans by Esterbauer et al. (1999), although this still has to be confirmed experimentally in the pig. An AT-rich region (5 187–5 211) is located downstream of this second polyadenylation signal in the porcine 3'UTR. (Adenylate and uridylate) AU-rich elements (ARE) in the 3'UTR mRNA are known to affect the stability, half-life and translation of the mRNA, depending on the protein which binds to the ARE (Barreau et al., 2005). Therefore, the presence of 2 polyadenylation signals and the location of the AT-rich region in between suggest a tight regulation of *PPARGC1A* mRNA turnover and protein synthesis in the pig. As for the promoter region, none of the discovered porcine SNPs in the 3'UTR are located in highly conserved parts of the sequence. This indicates that it is not likely that any of the SNPs have a major effect on the essential functions of *PPARGC1A*, but they may be involved in less critical changes, for instance in meat quality and carcass composition parameters.

As mentioned in the results section, due to low minor allele frequencies or technical difficulties

during the association analysis it was only possible to reliably assess whether SNP8, SNP10 and SNP12 had an impact on the tested traits. But because SNP6, SNP7 and SNP11 were always in complete linkage disequilibrium with SNP10, as seen in the initial mutation screening in multiple breeds, it is very likely that the data for these 3 SNPs can be deduced from the results for SNP10.

The *P*-values in Table 2 show that no significant associations with intramuscular fat percentage, leaf fat weight or last rib backfat depth were detected for SNP8, SNP10 or SNP12 in our study. However, this does not mean that none of the SNPs can have an influence on these 3 traits or on other meat quality and carcass composition characteristics. Several studies in the pig already found mutations in the coding region of *PPARGC1A* (exon 8 and 9) which were associated with multiple fat and feed characteristics (including our SNP12 and SNP13), some of these associations being population- or breed-specific (Jacobs et al., 2006; Stachowiak et al., 2007). Furthermore, in humans a gender-specific association with several obesity characteristics was found for a mutation in the 3'UTR of *PPARGC1A* (Esterbauer et al., 2002), which on the other hand was not detected in another human population (Vimalaswaran et al., 2006). The findings from this study indicate that further research of our newly detected polymorphisms in the regulatory regions of *PPARGC1A* and the ones in the coding sequence is necessary to evaluate their possible influence on *PPARGC1A* expression and their possible usefulness as future selection markers in the pig industry.

The new SNPs detected in this study could be of importance to the human research as well because of the high similarity between the pig and human genome, even in regard to SNP density (Grapes et al., 2006). Up till now, human *PPARGC1A* research mainly focussed on a p.Gly482Ser substitution and a few other SNPs in the coding region that have been associated with type II diabetes mellitus and body fat characteristics (Ek et al., 2001; Vimalaswaran et al., 2006). Therefore it would be interesting to verify whether these porcine SNPs are also present in humans and have an effect on *PPARGC1A* expression.

CONCLUSIONS

The results from this study contribute to our knowledge of the candidate gene *PPARGC1A* and

provide essential information on its sequence, necessary for unravelling the complex regulation of expression and functioning of *PPARGCIA* in the pig. Furthermore, the newly discovered SNPs in the regulatory regions can be used in future studies to (further) assess their usefulness as new selection criteria for improving meat quality while maintaining the leanness of the carcass as a whole.

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