

ORIGINAL ARTICLE

Investigation of two candidate genes for meat quality traits in a quantitative trait locus region on SSC6: the porcine short heterodimer partner and heart fatty acid binding protein genes

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Summary

A highly significant quantitative trait locus (QTL) on pig chromosome 6, affecting intramuscular fat (IMF), has previously been detected by our group and others. Two genes of positional and biological interest, the small heterodimer partner (SHP; NR0B2) and the heart fatty acid binding protein (FABP3; H-FABP), were investigated for meat quality traits and IMF respectively. SHP was partially sequenced (GenBank: DQ002896 and DQ002897) and mapped to the QTL region on porcine chromosome 6, affecting IMF. The map shows no recombination between SHP and FABP3, which was previously mapped to the same QTL region. Twelve single nucleotide polymorphisms were detected in the sequenced region of SHP gene. Haplotype information was used to investigate association between genetic variation and different meat quality traits. SHP haplotype combinations were found to have significant effect on connective tissue. However, further studies are needed to evaluate this possible association more effectively. The FABP3 is involved in fatty acid transport and has been studied as a candidate gene for IMF by several research groups. In our study, FABP3 genotypes were confirmed to be significantly associated with IMF in pigs. The average content of IMF in our population was 1.6%, which may indicate that the FABP3 polymorphism explains as much as 30–35% of the variation in IMF in our pig cross-population.

Introduction

In the production of meat there are many genes involved in metabolic processes that control growth and differentiation of the composite cells. Small heterodimer partner (SHP) is an atypical orphan nuclear receptor, which was shown to be expressed in liver, heart, pancreas (Seol et al. 1996) and adipose tissue (Nishizawa et al. 2002). SHP represses and inhibits the activity of liver X receptor (LXR) and retinoid X receptor (RXR; NR2B1), both of which were shown to have an important role in lipid

homeostasis (Chawla et al. 2001; Brendel et al. 2002). In another study SHP was shown to increase transcriptional activity of peroxisome proliferator-activated receptors (PPAR)- γ , which is a master regulator for adipocyte differentiation, and may act as an endogenous enhancer of it (Nishizawa et al. 2002). Kassam et al. (2001) also reported a positive effect of SHP in transcription of PPAR- α /RXR- α heterodimers. It means that any changes in the function of the SHP gene could indirectly influence the lipid homeostasis through the modification of the activity of RXR, LXR and PPAR- γ . Stulnig et al.

(2002) performed genome-wide gene expression profiling studies for LXRs. Based on their data, novel roles of LXR were presented including endocrine homeostasis and lipid metabolism. To note, LXR agonists were suggested to have an impact on overall protein metabolism as well, which makes SHP interesting as a candidate gene concerning other meat quality traits than those related to fat. SHP has been intensively studied in human but so far nothing has been reported for this gene in live-stock species. The human SHP gene has been localized to chromosome 1p36.1 (Lee et al. 1998), which is comparative with a quantitative trait locus (QTL) region in pig chromosome 6 affecting intramuscular fat (IMF) (Grindflek et al. 2001).

The heart fatty acid binding protein (FABP3; H-FABP) is involved in fatty acid transport from cell membrane to the intracellular sites of fatty acid utilization and is mainly expressed in cardiac and skeletal muscle (Veerkamp and Maatman 1995). FABP3 has previously been mapped to the QTL region on SSC6 (Gerbens et al. 1997; Grindflek et al. 2002) and genetic variants of FABP3 has been comprehensively studied by several groups (Gerbens et al. 1999, 2000, 2001; Nechtelberger et al. 2001; Urban et al. 2002; Øvilo et al. 2002).

The main aim of this study was to map the SHP gene to the pig genome, to detect single nucleotide polymorphisms (SNPs) in the gene, and to investigate whether the SHP gene variation is associated with pig meat quality investigated in a previous QTL study by Grindflek et al. (2001). Additionally, FABP3, which is located at the same position as SHP on SSC6, is investigated regarding to IMF in the same population.

Materials and methods

Animals and phenotypic data

A commercial Norwegian three-way pig cross were available for this study, consisting of half-sib families from five Norwegian-Landrace/Duroc sires each mated with eight Norwegian-Landrace/Yorkshire dams. For the SHP polymorphisms, three of the five families (151 individuals) were found to be informative and used for genotyping. For the FABP3 polymorphisms, all families (290 individuals) were found to be informative and used for further studies. Several carcass and meat quality traits useful for predicting the technological as well as eating quality of pork were recorded on the animals and this is described in more detail by Grindflek et al. (2001).

Polymerase chain reaction

Human SHP gene spans approximately 2 kb and consists of two exons and a 1321-bp intron. Primers were designed to amplify exon 1 and the intron, designed in the region of exon 1 (forward-1, reverse-1 and forward-2) and exon 2 (reverse-2) based on the human SHP sequence as follows: Ex1-F1 (5'-GGGGCCTGCCCATGCCAG-3'), Ex1-R1 (5'-GAGGATGGTCCCTTTCAG-3'), Ex1-F2 (5'-GGCCCAA-GACACTGTGACCTTC-3') and Ex2-R1 (5'-GGAATG-GACTTGAGGGTGGGA-3') respectively. The reaction volume was 25 μ l in both cases, containing 10 ng/ μ l of swine genomic DNA template, 10x polymerase chain reaction (PCR) buffer, 15 mM MgCl₂, 2 mM dNTP, 0.01 μ M of each primer and 5 U Gold Taq polymerase (Applied Biosystems, Foster City, CA, USA). To amplify the exon 1 and intron, PCR profile included an initial denaturation of 10 s at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at 61°C (exon 1) or 62°C (intron), 30 s (exon 1) or 2 min (intron) at 72°C and the final extension of 5 min at 73°C.

The PCR conditions used for amplifying fragments from the FABP3 gene are described by Gerbens et al. (1997).

Sequencing, restriction fragment length polymorphism screening and genetic linkage mapping

Sequencing of the PCR products was conducted for five boars (Duroc \times Landrace cross) using each of the forward and reverse primers. PCR products were purified from excess reaction components with Microcon-PCR device (Millipore Corp., Bedford, MA). Sequencing was performed on an ABI Prism 377 sequencer (Applied Biosystems) using ABI PRISM Big DyeTM Terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA). Sequencer Software 4.0 (Foster City, CA) was used to assemble the sequences and to identify polymorphisms. PCR restriction fragment length polymorphism (RFLP) was used for genotyping the animals for the chosen mutations. PCR product of SHP gene, obtained using primers Ex1-F2 and Ex2-R1, was approximately 1400 bp and was digested with the restriction enzymes *Hha*I, *Bst*NI and *Bsg*I (New England Biolabs, Beverly, MA, USA) to genotype the different SNPs. Fragments were separated by electrophoresis on 1.5% SeaKem LE Agarose gel (Cambrex Bio Science, Rockland, ME, USA). Polymorphisms of the cleavage site *Hha*I, *Bst*NI and *Bsg*I are shown in Figure 1. Linkage analysis was performed using the CRIMAP package, version 2.4 (Green et al. 1990). Markers

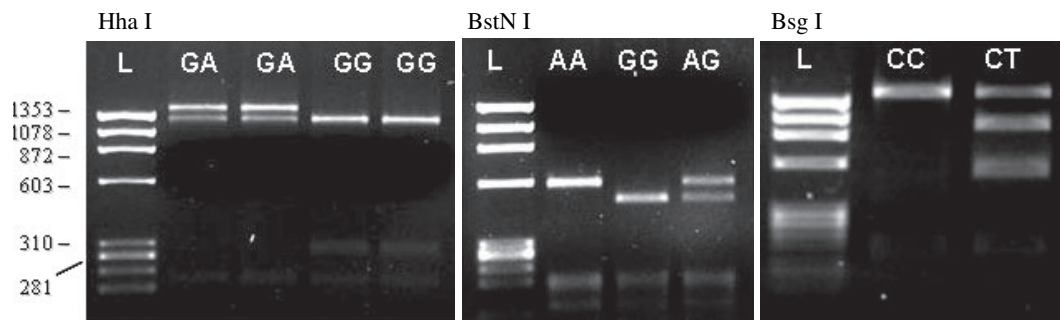


Figure 1 Polymorphisms of the cleavage site *Hha*I, *Bst*NI and *Bsg*I: Lane L is a molecular ladder ϕ x174; lane GA, GA, GG, GG, AA, GG, AG, CC and CT are the genotypes according to the substitution.

and genes used in a previous study by Grindflek et al. (2001, 2002) were included in the analysis.

To study the association between FABP3 gene and IMF we used RFLP markers described by Gerbens et al. (1997). Two *FABP3* SNPs were found to be polymorphic in our population and were used for genotyping the animals. For each *FABP3* RFLP test, the conditions of PCR and restriction conditions have been described by Gerbens et al. (1997). RFLP marker *FABP3a* was digested with the enzyme *Hae*III and *FABP3b* was digested with *Hin*fl. The undigested fragment of *FABP3a* is 683 bp and the digested fragment is 405/278, while the undigested fragment of *FABP3b* is 232 bp and the digested fragment is 173/59. In our study the allele H is the undigested fragment and allele h is the digested fragment in both RFLP tests (Table 1). Our estimation shows that the two polymorphisms, *FABP3a* and *FABP3b*, are in linkage disequilibrium.

Statistical analyses

The general linear model procedure was performed for the association study, using the statistical software package SAS Institute Inc., SAS/STAT Software (1996). Litter, sex and the SHP haplotypes were included in the analyses as fixed effects. Additionally, to ensure that the SHP haplotype effects were not confounded with selection in the sires, sire was included as fixed effect in the model. Slaughter weight was mostly used as a covariate, although the

genotype effect on IMF was analysed with a model including back fat thickness (BFT) traits as covariates instead of slaughter weight. The same model was used to analyse the *FABP3* genotypes effect on IMF. The *FABP3* polymorphisms (*FABP3a* and *FABP3b*) were analysed both together and separately. Different numbers of pigs were genotyped for SHP and *FABP3*, and the polymorphisms in the two genes were therefore not combined in haplotypes.

Results

Identification of polymorphisms in porcine SHP gene
Approximately 550- and 1400-bp long PCR products were obtained using primer pairs Ex1-F1/Ex1-R1 and Ex1-F2/Ex2-R1 respectively. Using both forward and reverse primers for sequencing, four different sequenced fragments of the SHP gene were obtained. The alignment of the 20 sequences altogether (four sequences from each of the five boars) resulted in two main fragments of the SHP gene, containing exon 1 plus part of the intron (GenBank: DQ002897) and part of the intron plus a short part of exon 2 (GenBank: DQ002896) respectively. Altogether, 12 SNPs were identified in the sequenced regions. One SNP was located in exon 1 without causing any amino acid changes in the protein sequence, and the rest of the SNPs were located in the intron. Only three of the five boars showed polymorphism for the 12 mutations found in the sequenced region of the SHP gene. Three of the 12

RFLP and IMF%	n	HH	n	Hh	n	hh	n	Sign. level
<i>FABP3a</i>	290	1.36 (0.12)	32	1.58 (0.06)	143	1.69 (0.07)	115	$p < 0.1$
<i>FABP3b</i>	290	1.11 (0.24)	9	1.55 (0.9)	97	1.65 (0.06)	183	$p < 0.05$

FABP3a was digested with the enzyme *Hae*III, and *FABP3b* was digested with *Hin*fl. Allele H is the undigested fragment and allele h is the digested fragment in both RFLP tests.

Table 1 Number of animals genotyped (n), and the mean value for intramuscular fat (IMF) and standard errors (between brackets) for fatty acid binding protein (*FABP3*) restriction fragment length polymorphism (RFLP) genotype classes

Table 2 Identified single nucleotide polymorphisms (SNPs) in the intron of the small heterodimer partner gene, which were used for haplotype studies

Primers	Restriction enzymes	Substitution	SNP position (GenBank)
Ex1-F2/Ex2-R1	<i>HhaI</i>	G/A	585 (DQ002896)
Ex1-F2/Ex2-R1	<i>BstNI</i>	A/G	798 (DQ002896)
Ex1-F2/Ex2-R1	<i>BsgI</i>	C/T	450 (DQ002897)

SNPs were chosen for further studies (Table 2). PCR-RFLP tests as it is described in the *Materials and methods* were used for genotyping. Four different haplotypes and seven haplotype combinations were constructed in the population studied: haplotype 1, GAC; haplotype 2, GAT; haplotype 3, GGC; haplotype 4, AGC; haplotype combinations: 11, 12, 13, 14, 23, 33 and 34.

Gene mapping

Haplotype information of 151 individuals was used to place the SHP gene to the pig chromosome 6 on the map made by Grindflek et al. (2001, 2002). Animals with haplotype combinations 23, 33 and 34 were excluded from the analyses because of extremely low frequency. The highest probability of SHP position was found to be located at the same position as SW1823 and the FABP3 (Figure 2).

Association study

SHP

The combined effects of the three substitutions were estimated as haplotype substitution effects on the recorded traits, and results from the association studies are given in Table 3. The SHP haplotype was shown to have significant effect on the connective tissue at a level of $p < 0.01$ and on the IMF and BFT (side) at a level of $p < 0.1$.

FABP3

The results from the association study of the FABP3a and FABP3b polymorphisms are given in Table 1. The analysis was performed separately for FABP3a and FABP3b in order to be able to compare the results with other studies (Gerbens et al. 1999; Nechtelberger et al. 2001; Urban et al. 2002; Òvilo et al. 2002) using the same RFLP tests. Significant variations in IMF were observed when analysing the FABP3a and FABP3b genotypes. Results show that allele h gives significant higher IMF in both markers, and the genetic effect seems to be clearly additive.

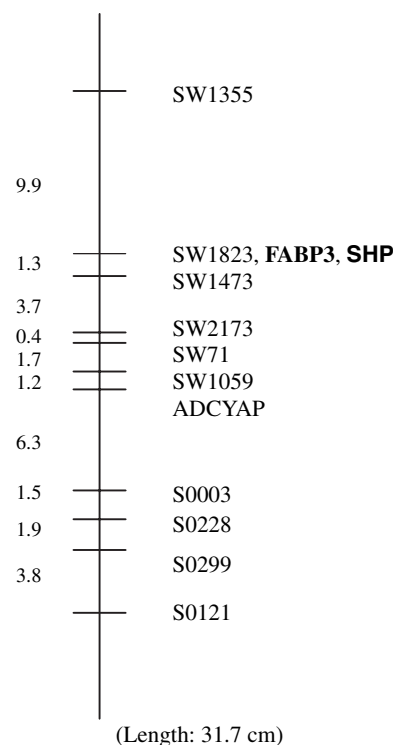


Figure 2 Linkage map for the porcine chromosome SSC6 containing the small heterodimer partner gene, placed on the map by Grindflek et al. (2001, 2002).

Additionally, contrasts between all combinations of FABP3a and FABP3b genotypes were analysed and the contrast between FABP3a-HH/FABP3b-HH and FABP3a-hh/FABP3b-hh was found to be the largest ($p < 0.0001$, results not shown), with least square mean values for IMF% of 1.2 and 2.0 respectively. FABP3 polymorphisms were also found to be associated with connective tissue at a significant level of $p < 0.1$ (results not shown).

Discussion

To date, no work has been carried out to investigate the role of SHP gene in different meat production traits in swine. In our study SHP was mapped to the QTL region of porcine SSC6 (Grindflek et al. 2001) and at the same position as FABP3, which has previously been comprehensively investigated as a positional and biological candidate gene for IMF (Gerbens et al. 1999, 2000, 2001; Nechtelberger et al. 2001; Urban et al. 2002; Òvilo et al. 2002).

Results from the association study between SHP haplotypes and the meat quality traits investigated, show that SHP is significantly ($p < 0.01$) affecting connective tissue in pigs (Table 2). Results from the

Haplotype combination	1/1	1/2	1/3	1/4	
No. animals	21	58	52	20	p-value
Traits					
IMF*	2.20 (0.19)	1.80 (0.11)	1.51 (0.11)	1.64 (0.19)	0.0705
PH	5.33 (0.038)	5.33 (0.021)	5.40 (0.023)	5.41 (0.038)	0.2693
CT**	2.08 (0.062)	2.17 (0.035)	2.035 (0.037)	2.18 (0.062)	0.0059
Protein	22.47 (0.16)	22.50 (0.09)	22.52 (0.10)	22.56 (0.16)	0.9903
WBC	6.48 (0.35)	6.52 (0.19)	6.69 (0.21)	6.35 (0.35)	0.7348
BFT					
Shoulder	16.5 (1.29)	15.70 (0.72)	13.37 (0.77)	13.89 (1.29)	0.2501
Side*	18.34 (1.12)	16.29 (0.62)	14.34 (0.67)	14.02 (1.12)	0.0742
Loin	12.48 (0.99)	11.21 (0.59)	10.63 (0.59)	11.12 (0.99)	0.4909
Warmwht	80.21 (3.62)	79.56 (2.03)	76.12 (2.17)	79.16 (3.16)	0.6214
Endwht	110.43 (1.00)	110.16 (0.56)	110.99 (0.60)	110.55 (1.00)	0.7955

IMF, intra muscular fat; WBC, water-binding capacity; BFT, back fat thickness; CT, connective tissue Warmwht, warm weight; Endwht, end weight.

Values are given as mean (SD). The estimated standard errors are given in parentheses.

**p < 0.01; *p < 0.1.

Table 3 Effect of small heterodimer partner haplotypes for different meat quality in the commercial Norwegian three-way pig cross population

analysis of FABP3a and FABP3b genotypes, also revealed significant ($p < 0.05$) association with connective tissue (results not shown), probably because of the complete linkage disequilibrium between the genes in this study. Interestingly, Stulnig et al. (2002) found that SHP forms heterodimer with LXR and represses its activity, which agonists were suggested to have an impact on connective tissue architecture. It is, however, important to note that association observed may arise in three-way cross-population where considerable linkage disequilibrium exists. Furthermore, very few animals were available in this study and also with several traits analysed we might expect by chance that significant associations occur purely by chance. Notably, however, is that haplotype information was used instead of single SNP that can provide more information on the complex relationship between DNA variation and phenotypes than any single SNP (Stephens et al. 2001), and that FABP3 genotypes also show significant association with connective tissue.

Significant associations were observed between IMF and both FABP3a and FABP3b genotypes (Table 1), which are in agreement with other studies (Gerbens et al. 1999, 2000). The contrast between the homozygous genotypes of FABP3 markers was 0.4–0.5% IMF (Table 1), which is very similar to the results obtained by Gerbens et al. (1999). The average content of IMF in our population was 1.6%, which may indicate that the polymorphism explains as much as 30–35% of the variation in IMF. Other studies, however, do not show clear association between genetic variance of FABP3 and IMF (Nech-

telberger et al. 2001; Urban et al. 2002; Òvilo et al. 2002), and conclude that FABP3 does not include the causal mutation. Association between IMF and BFT (side) and the different SHP haplotype combinations were also close to significant ($p = 0.07$). In our study the results are quite clear, but it is however, important to note the concerns regarding to linkage disequilibrium mentioned above. It is therefore still not possible to draw any clear conclusion based on these results.

It should also be noted that small differences was observed in the significance level of the association between IMF and connective tissue and the two genes. These differences might have occurred due to the limited number of animals used in this study, especially in the study of SHP. Furthermore, the SNPs can originate from different time of evolution resulting in different association to the traits, if the polymorphisms are not the causative ones.

In this study, we confirm the FABP3s association with IMF in pigs. Because of the contrary results in the literature, FABP3 polymorphisms are most likely not the causal mutation of the effect observed. It might, however, have a practical interest as a marker for pig breeding programs aimed at improving IMF. Regarding the new candidate gene SHP, further studies are needed to evaluate the possible association between SHP and the traits connective tissue and IMF more effectively. These data may be too preliminary to allow us to distinguish between the possible explanations: false-positive association, effects of linked mutations in SHP and effects of linked genes.

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