Association of parathyroid hormone-like hormone (PTHLH) and its receptor (PTHR1) with the number of functional and inverted teats in pigs

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Introduction

Many dam line breeding programmes in pig populations focus on udder quality. In particular, the teat number and functional mammary gland capability are important selection criteria for increasing the survival rate of piglets. The most frequent and economically relevant inherited disorder of the mammary complex in pigs is the inverted teat, resulting in non-functional teats that cannot be suckled by the offspring. The defect occurs in commercial pig breeds with frequencies between 7.6% and 30% (Niggemayer 1993; Brevern et al. 1994; Mayer & Pirchner 1995; Jonas et al. 2008). Wiesner

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Family-based association; inherited disorder; liability trait; mammary gland; mothering ability.

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Summary

Parathyroid hormone-like hormone gene (PTHLH) and its receptor, parathyroid hormone/parathyroid hormone-like hormone receptor 1 (PTHR1), play a role in epithelial mesenchymal interactions during growth and differentiation of different tissues and anatomic structures, including teats. Therefore, PTHLH and PTHR1 were evaluated as functional candidate genes for their effects on number and shape of teats in pigs. In particular, focus was on the occurrence and number of inverted teats, the most frequent and economically relevant teat developmental defect in pigs. For this purpose, association and linkage of the PTHLH gene and the PTHR1 gene with inverted teat defect and the total number of teats and inverted teats were studied in an experimental Duroc and Berlin Miniature pig (DUMI) population. Polymorphism C1819T of PTHLH was significantly associated with inverted teat phenotype (p = 0.014), total number of teats (p = 0.047) and was close to significance with the number of inverted teats (p = 0.078). Polymorphism C375T of PTHLH was close to significance with the inverted teat phenotype (p = 0.122) and showed no significant association with the total number of teats (p = 0.621) and the number of inverted teats (p = 0.256) in the DUMI population. Association analyses were also performed for combined effects of PTHLH and PTHR1 in order to address potential interaction, however, revealed no indication of effects of interaction. The function, position and the association shown here promote PTHR1 as a candidate gene for number of teats and in particular for affection by and number of inverted teats.

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Association of porcine PTHLH and PTHR1 with teat traits

S. Tetzlaff et al.

and Willer (1978) considered a complex inheritance of the liability to develop the defect with the presence of a major gene and several minor genes causing this defect. However, the mode of inheritance and the number of genes involved are still unknown. Heritability was estimated to be between 0.2 and 0.5 (Mayer 1994). In a Duroc and Berlin Miniature pig (DUMI) population, 53.6% suffered from mammary gland abnormalities, 42.2% had inverted teats and 17.9% showed extra teats (Hardge et al. 1999).

Parathyroid hormone-like hormone gene (PTHLH) and the parathyroid hormone/parathyroid hormone-like hormone receptor 1 (PTHR1) were shown to regulate epithelial mesenchymal interactions during the formation of the mammary gland (Foley et al. 2001). Therefore, PTHLH and PTHR1 are functional candidate genes for traits related to mammary gland and teat development. The aim of this study was to investigate the association and linkage of PTHLH and PTHR1 gene polymorphisms with the inverted teat defect.

Materials and methods

Animals and phenotypes

Animals (n = 313) of an experimental population based on a reciprocal cross of the DUMI population were used. At 200 days of age, two investigators of a team of four trained and skilled persons observed the phenotypes of the teat traits involved in this study. The animals were placed on their backs and teats were evaluated by inspection and palpation. The numbers of functional and inverted teats were recorded. Animals without inverted teats were classified as ‘non-affected’ animals while those with at least one inverted teat were categorized as ‘affected’.

Genotypes

The PTHLH polymorphism analysis was performed by an allelic discrimination assay (Assays-by-DesignSM Service; ABI PRISM® 7000; PE Applied Biosystems, Darmstadt, Germany) using primers PTHLP-FW: 5’-GAGCGTCGCGGTGTC-3’ and PTHLP-RW: 5’-AGCGCCCGCAGGAG-3’ for polymerase chain reaction (PCR) amplification and allele-specific probes PTHLP-V2: 5’-VIC-CTGAGCTATTCGGTGCC-TAMRA-3’ and PTHLP-M2: 5’-FAM-CTGAGCTAT-TTCTGCCC-TAMRA-3’ for discrimination of either C or T at nt 375 of PTHLH (GenBank accession no. AY193782; Chomdej et al. 2004). PTHR1 polymorphism analysis was conducted by restriction fragment length polymorphism assay. To amplify the region containing a PTHR1 single nucleotide polymorphism (SNP) primers PTHR-F: 5’-GCTATGGTCCAGATGTTCT-3’ and PTHR-R: 5’-ACTGTCTCCACACTCTCCTG-3’ were used and PCR fragments were subsequently incubated with the restriction enzyme MboII (Fermentas, St. Leon-Rot, Germany) suitable to discriminate the C and the T alleles at nt 1819 (GenBank accession no. NM_214382; Tetzlaff et al. 2007). Amplification reactions were conducted in a final volume of 15 µl, containing 0.5 unit of Taq DNA polymerase, 0.2 mM of each dNTP, 0.2 μM of each primer, 1x buffer according to the manufacturer’s instruction (GeneCraft, Cologne, Germany) and 50 ng of DNA.

Expression

In order to survey expression of porcine PTHLH and PTHR1 genes in different tissues and organs, quantitative reverse transcriptase PCR (qRT-PCR) was performed using the LightCycler® 480 system (Roche, Penzberg, Germany). Total RNA was isolated from epithelial teat tissue, connective teat tissue, liver, kidney, adrenal gland, spleen, tonsil, lymph node, muscle, hypothalamus and pituitary from adult pigs of German Landrace using Tri-Reagent (Sigma, Taufkirchen, Germany) and NucleoSpin® RNA II kit (Macherey-Nagel, Düren Germany) including DNase treatment following the manufacturer’s instructions. RNA samples were visualized on 1.5% formaldehyde containing agarose gel to check the integrity and the concentration was measured by spectrometry with a NanoDrop® ND-1000 spectrophotometer (PEQLAB).

First-strand cDNA was synthesized from 1 μg of total RNA using random primers and oligo (dT)13N in the presence of Superscript™ III reverse transcriptase (Invitrogen, Hamburg, Germany). The primers for PTHLH, PTHR1 and ribosomal protein L32 (RPL32) as

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequence</th>
<th>Annealing (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTHLH</td>
<td>GCAAGAGCACGGAAAAAAAGAGAGCGATGGGGAGACATTG</td>
<td>64</td>
<td>261</td>
</tr>
<tr>
<td></td>
<td>AGACAAATGGGGGAGACAGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTHR</td>
<td>TGGCTCTCTTGGCTCTCTGGTCTTACTGAGCCACACGC</td>
<td>60</td>
<td>178</td>
</tr>
<tr>
<td>RPL32</td>
<td>AGGCGAGATCGCTAAAGAGTGGTTGCTCTCATACCAAAGTT</td>
<td>60</td>
<td>165</td>
</tr>
</tbody>
</table>
an internal reference were described in Table 1. The reactions were performed in a final volume of 12 μl using 6.0 μl of LightCycler® 480 SYBR Green I Master (Roche), 600 nM of each primer and 100 ng of cDNA. Amplification conditions were 95°C for 10 min, 40 cycles of 95°C for 15 s, annealing temperature (Table 1) for 10 s and 72°C for 15 s. At the completion of the amplification protocol, all samples were subjected to a melting curve to verify the absence of any non-specific products.

Statistics

In order to evaluate the genes for association and linkage with total number of teats, number of inverted teats and the affection status of the Family-Based Association Test (FBAT) was used in the experimental DUMI population with the empirical variance option and the null hypothesis stating ‘no association, but linkage’ (FBAT: http://www.biostat.harvard.edu/~fbat/default.html). The quantitative association analyses were performed in bi-allelic tests under the condition of an additive genetic model (Horvath et al. 2001). A statistical evaluation of the interaction between PTHLH and PTHR1 gene polymorphisms and their influence on inverted teat trait was executed with LOGISTIC procedure of the SAS software package 9.1 (Cary, NC, USA) using father, mother and gender as additional explanatory variables of interest in the model.

Results

Genotype analysis

Two non-synonymous C>T SNP were detected at nucleotide position 375 (S19L) of the porcine PTHLH cDNA and at nucleotide position 1819 (L556F) of the porcine PTHR1 cDNA (Chomdej et al. 2004; Tetzlaff et al. 2007). Mendelian inheritance of these polymorphic sites was monitored in individuals of the experimental DUMI population. The frequencies of genotype combinations are presented in Table 2.

Expression of porcine PTHLH and PTHR1 genes

The qRT-PCR of 11 tissues including epithelial and connective teat tissues from adult pigs indicated differential expression of porcine PTHLH and PTHR1 genes as shown in Figure 1. The PTHLH gene was expressed in multiple tissues thereby clearly detectable in epithelial and connective teat tissues. A lower expression was detected in the kidney and adrenal gland. The expression pattern of PTHR1 gene revealed that liver and kidney were the major sites of expression. Low abundance was observed in the muscle and pituitary.

Association with inverted teat trait

Association analyses were first performed separately for each locus. The results of SNP C375T of PTHLH and C1819T of PTHR1 with inverted teat as the affection trait are summarized in Table 3. SNP C1819T of PTHR1 gene was significantly associated with total teat number (p = 0.047), the affection trait (p = 0.013) and close to significance with total inverted teat number (p = 0.078). SNP C375T of PTHLH showed no significant association with total teat number (p = 0.621) and total inverted teat number (p = 0.256) but was close to significance with inverted teat as the affection trait (p = 0.122).

Discussion

This investigation is the first to attend to association and linkage of PTHLH and its receptor PTHR1 with the inverted teat defect in pigs. The emergence of inverted teats depends on insufficient mesenchymal...
proliferation at the teat ground during teat development. As far as proliferation processes are concerned, the defect of inverted teats is related to the trait of the number of teats. The number of teats may also depend on local signalling between adjacent embryonic tissues (Jonas et al. 2008). Foley et al. (2001) elucidated the influence of PTHLH and PTHR1 on the regulative interaction of epithelial mesenchymal proliferation during the formation of the mammary gland. Prior experiments with knock-out mice indicated involvement of PTHLH in the ontogenesis of a functional mammary gland, because these mice developed the inverted teat phenotype (Wysolmerski et al. 1998; Dunbar et al. 2001). Furthermore, the expression results of both genes in relevant tissues for the inverted teat defect approved the results reported in other species (Kong et al. 1994; Kobayashi et al. 2005). Thus, PTHLH and PTHR1 genes were contemplated as functional positional candidate genes for the inverted teat phenotype. Our results for the SNP C1819T in PTHR1 have revealed a significant association and linkage to teat number as well as occurrence of inverted teats. However, association with teat number as well as inverted teat defect and the SNP C375T in PTHLH could not be verified. Furthermore, the amino acid exchanges of both SNP were analysed by SIFT (Sorting Intolerant from Tolerant; http://blocks.fhcrc.org/sift/SIFT.html). SIFT is based on the premise that important amino acids will be conserved among sequences in a protein family, so changes at amino acid conserved in the family should affect protein function (Ng & Henikoff 2002). Thus, phylogenetic relationships do not promote functional impact of the amino acid exchanges. However, the amino acid exchanges are between polar (serin) and neutral (leucine) and neutral (leucine) and aromatic (phenylalanine) amino acids, respectively, therefore impact on structural and biochemical properties of the proteins cannot be ruled out. For PTHLH, the polymorphism is located in its signal peptide that directs post-translational transport. The SNP at nt 1819 of PTHR1 is located in its intracellular tail, which is involved in signal transduction. SNP in the coding regions of the genes are not likely to affect their expression. Any functional effects have to be confirmed. When analysing genes of hormones and their receptors effects of interaction can be expected, however no interaction between PTHLH and PTHR1 genotypes were observed. That might be because of the limited number of animals. PTHLH was mapped slightly distal of quantitative trait loci (QTL) for number of nipples and the inverted teat defect on SSC5 (Lee et al. 2003; Chomdej et al. 2004; Rodríguez et al. 2005; Jonas et al. 2008), whereas PTHR1 was displayed in close proximity to a QTL for the inverted teat defect on SSC13 (Tetzlaff et al. 2007; Jonas et al. 2008). The SNP in PTHR1 likely is a marker in close linkage disequilibrium to a causative polymorphism affecting the liability for the inverted teat defect; PTHR1 even represents a good candidate for the causal polymorphism. The SNP of both genes were found to segregate among pigs of commercial breeds, German Landrace, Large White, Pietrain, Hampshire and Duroc, with allele ‘C’ at both SNP being the prominent one (Chomdej et al. 2004; Tetzlaff et al. 2007). Effects of both genes on teat phenotypes but also on other traits will be evaluated in animals of commercial dam lines in order to further

**Table 3** Association of PTHLH and PTHR1 with the affection by inverted teats (AF), total number of inverted teats (TIT) and total number of teats (TT) in the DUMI resource population as revealed by FBAT analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>allele</th>
<th>Freq</th>
<th>p</th>
<th>p</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTHLH(C375T)</td>
<td>C</td>
<td>0.5</td>
<td>0.122</td>
<td>0.621</td>
<td>0.256</td>
</tr>
<tr>
<td>PTHR1(C1819T)</td>
<td>C</td>
<td>0.6</td>
<td>0.013</td>
<td>0.047</td>
<td>0.078</td>
</tr>
</tbody>
</table>

**Figure 1** Tissue-specific expression pattern of PTHLH and PTHR1 genes assayed by qRT-PCR. Copy number of RPL32 gene was measured to normalize for equal RNA amounts. 1, epithelial teat tissue; 2, connective teat tissue; 3, liver; 4, kidney; 5, adrenal gland; 6, spleen; 7, tonsil; 8, lymph node; 9, muscle; 10, hypothalamus; 11, pituitary.
qualify the genes as functional positional candidate genes in a gene-assisted selection. Further analyses of trait association in other populations as well as functional assays will provide more insight into the causal nature of the polymorphisms.

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