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Verification of chromosomal regions affecting the inverted teat development and their derivable candidate genes in pigs

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Introduction

The inverted teat defect is the most common and most important disorder of the teat in pigs (Blendl et al. 1980, Brevern et al. 1994). It occurs in different commercial pig breeds with frequencies between 8 to 30% (Blendl et al. 1980, Hittel 1984, Mayer and Pirchner 1995). The detected heritability estimates ranging from 0.2 to 0.5 depending on the population and statistical model used (Brevern et al. 1994, Mayer 1994). The inheritance is not fully understood and the number of genes involved is unknown. Different studies reported twenty-five QTL for number of teats on different chromosomes in pigs, but there are no results for traits of the udder quality in pigs (Beeckmann et al. 2003a, Beeckmann et al. 2003b, Cassady et al. 2001, Cepica et al. 2003, Dragos-Wendrich et al. 2003, Hirooka et al. 2001, King et al. 2003, Rohrer 2000, Yue et al. 2003).

The objective of this study was to identify chromosomal regions affecting the inverted teat defect by linkage mapping in an experimental F2 population, to investigate positional and physiological candidate genes, to validate these genes using association analysis and to verify the results of linkage and association analysis in animals of dam breeds.

We have identified a number of genes to be candidates for mammary gland/ teat development and growth.

The *relaxin 1* (*RLN1*) gene is involved in a number of functions in mammals, including the stimulation of tissue growth, differentiation and remodeling during pregnancy (Sherwood 2004). This hormone is important for the function of reproductive tissues, heart, kidney, and brain. It has a number of functions in mammals that are generally associated with female reproductive tract physiology (Bathgate et al. 2002). RLN1 was assigned to Sscr1q28-29 by physical mapping (Ellegren et al. 1994).

The *leucin-rich repeat-containing G protein-coupled receptor 7* (*LGR7*) was recently identified as the only receptor of *RLN1* (Hsu *et* al. 2000, Hsu et al. 2002, Muda et al. 2005). Similar to known physiology of *RLN1, LGR7* was found to be expressed in stroma cells of endometrium and breast. Different alternative splicing forms were detected in *LGR7* (Muda et al. 2005, Scott et al. 2005).

Growth factors are involved in intercellular signaling of the glands. The best seen effect of the *growth hormone* (sommatotropin, *GH*) is the morphogenesis of the mammary ducts. The growth hormone is lactogene in many species, for this the presence of *GH* receptor in the epithelial cell of the teat is necessary. Within the lactation, *GH* support the secretion of high-energy milk with a high fat content by direct action on the mammary gland (Flint and Gardner 1994). *GH* was mapped on chromosome 12p14 in pig (Thomsen et al. 1990).

The *transforming growth factor beta 1* (*TGFB1*) belongs to the family of growth factors. The *TGFB* superfamily consists of the factors *TGFB1*, *TGFB2* and *TGFB3*. Both *TGFA* and *TGFB* could be shown to influence the regulation of teat growth and differentiation of mammary gland (Oka et al. 1991).

Material and methods

Animals. One hundred pigs of different dam breeds (German Landrace, German Large White and their crossbreeds) from different breeding companies were used in this study. Additional four hundred animals of the experimental population based on a reciprocal cross between Duroc and Berlin Miniature pig (DUMI population) were also used in this study. DNA for genotyping was isolated using the phenol-chloroform extraction.

QTL study. For performing QTL analysis eighty loci covering the porcine autosomes with mean interval of 27.9 cM were selected from published linkage maps (USDA-MARC and PiGMaP). Standard PCR was performed at 94°C for 3 min, followed by 30 to 40 cycles at 94°C for 1 min, 55°C to 65°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The annealing temperature was used depending on the optimal temperature for each primer. Electrophoresis was performed with Li-Cor Sequencer System (Li-Cor model 4200 automated DNA sequencer) using 12% SequaGel in 1xTBE buffer at a power of 1500V, 50mA, 50W and 50°C temperature.
Microsatellite genotyping results were analyzed by software OneDScan, version 4.10 (Li-COR Biotechnology). The program CRIMAP, version 2.4 was used for mapping of the markers (Green 1992). The QTL analyses were performed with the software package Genehunter, Version *2.0 (Whitehead Institute, Cambridge, Massachusetts, USA)* (Kruglyak et al. 1996)

Genotyping. To genotype polymorphisms within porcine *relaxin 1 (RLN1)* gene, specific primers were used based on the published sequences of *RLN1* (Wimmers et al. 2002a). An SSCP protocol was established for the polymorphism detected in exon 1 of *RLN1* gene. Products for SSCP were diluted 1:1 with loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol), denatured at 95°C for 5 min, chilled on ice and loaded on 12% polyacrylamide gels (acrylamide:bisacrylamide 49:1). Gels were run at 12 W for 2.30 hours at room temperature in 0.5 × TBE. Banding patterns were detected by silver staining. For genotyping of the SNP in the intron of *RLN1*, an RFLP using *Cfo*I enzyme was performed.
The SNP of *TGFB1* gene was genotyped in 400 animals of the F2-DUMI resource population, and 100 commercial animals based on the affected sibpair design. SSCP protocol was also established being diagnostic for the polymorphism detected in *transforming growth factor beta 1* (*TGFB1*) gene (Wimmers et al. 2002b).
A pair of primers designed by Larsen and Nielsen (1993) was used to genotype the *growth hormone (GH)* gene. An 605 bp PCR amplicon was digested with restriction enzymes *Dde*I and *Apa*I, respectively by incubation at 37°C overnight. The products were vizualised in 2.5% agarose gels stained with ethidium bromide and photographed under UV light.
In this study the FBAT program (Version 1.4) was used to perform the qualitative and quantitative family-based analyses. All analyses were done under the condition of an additive model (Horvath et al. 2001).

Mapping of candidate genes. Animals of the experimental population were genotyped at the *RLN, GH,* and *TGFB1* locus. Subsequently twopoint and multiple procedures of the CRIMAP package (version 2.4) were used for linkage mapping (Green et al. 1990).

The genetic mapping of *leucin-rich repeat-containing G protein-coupled receptor 7* (*LGR7*) was performed by radiation hybrid mapping. The porcine INRA-university of Minnesota porcine radiation hybrid (IMpRH) mapping panel (Yerle *et al.* 1998) was used for mapping. The statistical analysis for placement of markers was done with the whole RH data set by using the MultiMap software (http://imprh.toulouse.inra.fr/).

Gene characterization. In order to characterize the porcine *LGR7* gene, RNA was isolated from piglet testis using TRI Reagent (Sigma-Aldrich, Munich, Germany) and DNase-treated (Qiagen, Hilden, Germany). The cDNA sequence of *LGR7* was determined by 5`RACE and 3`RACE (SMART^{TM} RACE cDNA Amplification Kit, Clontech, Heidelberg, Germany). Sequencing was performed using different primers derived from ESTs found with BLAST.
In order to confirm mRNA expression of *LGR7*, primers to amplify different fragments within the first and last part of the coding region were designed. Total RNA from different tissues of male and female pigs was DNase-treated (Qiagen, Hilden, Germany), and used to generate single-stranded cDNAs using Superscript first-strand synthesis system (Invitrogen, Karlsruhe, Germany) according to the manufacturer`s instructions. RNA (200ng) was primed for cDNA synthesis using a hexamer primer (500ng/ml). Expression analysis was done by semi-quantitative RT-PCR. The 18S ribosomal RNA was used as internal reference. Different fragments were used to identify splicing variants. Amplicons were sequenced by CEQ8000 using DTCS Quick Start Mix (Beckman Coulter, Krefeld, Germany).

Results
QTL mapping revealed the highest NPL value for the inverted teat defect on SSC6 in both populations (NPL-score in DUMI and commercial population 9.25 and 1.64, respectively). Significant p-values were also detected on SSC1 in both population, the result could be confirmed by association analysis using FBAT. The results of SSC8 give good evidence for suggestive QTL in DUMI but not in the commercial population. A significant QTL was also found on SSC12 in DUMI. All results of linkage mapping could be confirmed by association analysis using the individual markers.
Among the 384 F2 animals of the DUMI families that were genotyped for *RLN1* gene frequencies of the ‘a’ and ‘c’ alleles were 0.29 and 0.71, respectively. The SNP was found to segregate among pigs of three commercial breeds with frequencies of the ‘a’ and ‘c’ alleles in the 22 families following the affected sibpair design were 0.13 and 0.87, respectively. For the other transversion (T > G) at position 9 of the intron 1, frequencies of the ‘g’ and ‘t’ alleles were 0.48 and 0.52, respectively, in the F2 DUMI population. In the animals of the commercial families frequencies of the ‘g’ and ‘t’ alleles were 0.91 and 0.09, respectively. This study found significant evidence of family-based association in *RLN1* (T9intG) loci ($Z = 2.762$, $p = 0.0057$) with inverted teat defect affection in the DUMI population. No significant differences were detected for *RLN1* (A22C) loci with the affection. High significant association was also found between *RLN1* loci and number of teat ($p < 0.05$) and number of inverted teat ($p < 0.05$). In the commercial population, no significant differences were detected in the affecting status in both *RLN1* (T9intG), *RLN1* (A22C) loci separately. Results of linkage mapping confirmed findings of *RLN1* mapped on SSC1.
The porcine *LGR7* cDNA was 2414 bp in length with a 136 bp 5`-untranslated region, encoding for approximately 759 amino acids. The most significantly linked marker was SW368 on chromosome 8 (distance = 28cR; LOD = 14.04), indicating the location of *LGR7* on the SSC8. The human *LGR7* gene has been mapped to chromosome 4q32.1, the comparative region to SSC8. *LGR7* in pig is highly expressed in heart, lung, brain, skin, uterus and teat. Different alternative splicing variants were detected in the gene by comparative sequencing, revealing more than three *LGR7* variants within 5`-

UTR region to exon 5 and at least one alternative form between exon 16 to exon 18. Translation of some variants may lead to a stop codon.

The A/G transition in exon 5 at position 797 of the *TGFB1* gene was observed in 21 families of DUMI population and Mendelian inheritance of the alleles could be demonstrated. Frequencies of the allele 'g' and 'a' were 0.7 and 0.3, respectively. The SNP was genotyped in 100 commercial animals where frequencies of the allele 'g' and 'a' were 0.98 and 0.02, respectively. No significant differences were detected in the affection and *TGFB1* (A797G) loci. High significant association was found with the number of teat ($p < 0.05$) and the number of inverted teat ($p < 0.05$). The affection was associated with *TGFB1* (A797G) loci ($Z = 2.252$, $p = 0.02431$). These results could be confirmed in the DL and the crossbreed animals. *TGFB1* linkage mapping using CRIMAP package version 2.4, revealed close linkage to loci S0300, SW193 and SW1067 (distances 7.3 cM, 13.8 cM, 24.5 cM; LOD scores 44.8, 19.2 and 21.4). Our results are in agreement with published genetic and physical map.

For *GH,* two alleles were generated. Significant association was only found for the genotypes of the animals of the DUMI population for both polymorphisms with p-values of 0.016 and 0.013, respectively.

Future prospects for investigation of candidate genes for inverted teat defect in pigs

This study was performed to demonstrate the association of SNP in *RLN*, *TGFB1*, and *GH* genes with the inverted teat defect in pigs and to conduct a linkage analysis in two populations for the inverted teat defect. Results of association analysis could verify the QTL found in experimental and commercial populations.

Additional expression difference between normal and inverted teat in combination with the analysis of different development stages using microarray analysis may lead to a better understanding of the involvement of genes to the teat quality in pigs, in addition to the finding of already conducted linkage and candidate gene approaches.

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