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Characterization of Relaxin-3/INSL7 and its receptor being candidate genes for the inverted teat defect in pigs

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Introduction

The relaxin-like peptide family belongs to the relaxin/insulin superfamily. This superfamily consists of relaxin-like peptides including *RLN1*, *RLN2*, and *RLN3/INSL7*, and insulin-like peptides comprising *INSL3*, *INSL4*, *INSL5*, and *INSL6*. *RLN* is know as a hormone involved in a number of functions in mammals including the stimulation of tissue growth, differentiation, and remodeling during pregnancy that are generally associated with female reproductive tract physiology (Sherwood 1994). *RLN* increases growth and differentiation of the mammary gland and nipple and induces the breakdown of collagen, one of the main components of connective tissue (Unemori and Amento 1990). Sequences of *RLN* of many species have been characterised. All known variants of *RLN* have a two-chain structure comprising an A- and B-peptide chain linked by disulfide bonds with an intrachain disulfide bond in the A-chain (Bathgate et al. 2002). The *RLN3* gene, the newest known member of the relaxin/insulin superfamily (Bathgate et al. 2002), has been recently reported as a ligand for two related orphan G protein-coupled receptors (*GPCRs*), *GPCR135* and *GPCR142* (Bathgate et al. 2002, Liu et al. 2003a, Liu et al. 2003b, Chen et al. 2005). Pharmacological studies of *GPCR135* gene indicated that *RLN3* is the only member of this family being able to activate *GPCR135* gene. The *RLN1* gene was proposed to be tested for association in a positional functional candidate gene approach. This gene could be mapped on SSC1 in the region where QTL for number of teats and the inverted teat defect could be found (Rohrer 2000, Cassady et al. 2001, Ün 2002, Wimmers et al. 2002, Beeckman et al. 2003, Oltmanns 2003). Female mice lacking a functionally active *RLN1* gene failed to relax and elongate the interpubic ligament of the pubic symphysis and could not suckle their pups. This is evidence for the finding that *RLN1* is involved in proliferation and differentiation processes of the mammary gland (Zhao et al. 1999). A number of QTL could be detected for number of teat and for the inverted teat defect (Hirooka et al. 2001, Ün 2002, Lee et al. 2003, Oltmanns 2003). Due to the finding in these studies giving evidence of interesting chromosomal regions for the trait of teat development also on SSC2, *RLN3* was proposed as functional and positional candidate gene for the inverted teat defect. The objectives of this study were to characterize *RLN3* and its receptor *GPCR135* gene in pigs, to determine the chromosomal location and to evaluate the association of *RLN3* and *GPCR135* gene with the inverted teat defect in pigs.

Material and Methods

Animals. The experimental pigs used were from a three generation F2 resource population which was established by reciprocal crossing of Berlin Miniature Pig and Duroc (DUMI) at the Institute of Animal Science of the Humboldt University in Berlin (Hardge et al. 1999). Animals of the F2 generation were kept and performance tested at the research farm Frankenforst of the Institute of Animal Science in Bonn. To confirm the results from the experimental population, animals of a commercial population were used. Samples of German Landrace (DL) and German Large White (DE) pigs and their crossbreeds were collected from testing stations and different farms, teats traits were evaluated at slaughterhouse. These animals were used due to the fact, that DL and DE are the main dam breeds in German breeding systems.

Linkage mapping. Animals of F2 DUMI resource population were genotyped at the *RLN3* and *GPCR135* gene locus. Subsequently twopoint and multiple procedures of the CRIMAP package (version 2.4) were used (Green et al. 1992). *RLN3* and *GPCR135* gene linkage mapping was performed against four microsatellite markers on chromosome 2 and chromosome 16, respectively.

Expression analysis. Total RNA was isolated from different tissue of two different male pigs (muscle, heart, spleen, lymph nodes, skin, brain, teat, lung, testis, tonsil, liver and kidney) and one sow (uterus and inverted teat) using Trizol® (Gibco BRL). First strand cDNA was synthesed from 1 ug of total RNA using random primer and oligo (dT)12N primer in the present of reverse transcriptase (Superscript II, invitrogen). The expression pattern of *RLN3* and *GPCR135* was analyzed in different tissues following the protocol for semi-quantitative RT-PCR. The expression of *18S* rRNA was used as an internal reference.

SNP screening. Porcine *RLN3* gene sequences available from GeneBank accession number AB076661 and the software Primer3 available from internet (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were used. Different primers were designed to sequence the *RLN3* gene and to screen for polymorphism. Genomic DNA sequence of the porcine *GPCR135* gene was obtained starting with heterologous primers designed from conserved regions of the human, mouse and rat *GPCR135* genes and subsequent using homologous primers.

Genotyping and association analysis. A 434-bp fragment was amplified within *RLN3* gene to perform PCR-RFLP analyse using *Rsa*I endonuclease. A 334-bp fragment of *RLN3* gene was used in PCR-RFLP analyses using *Taq*I endonuclease. The *GPCR135* gene was genotyped by PCR-RFLP using *Msp*I restriction enzyme. Digestion of the resulting 859 bp product with *Msp*I revealed a polymorphism with two alleles. A 198-bp fragment was amplified to genotype the other SNP detected in GPCR135 gene by PCR-RFLP using *Mnl*I endonuclease. The PCR and PCR-RFLP products were run in 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

The Family-Based Association Test (FBAT) (version 1.4) was used to perform the association analysis of the genotypes with the teat characteristics (Horvath et al. 2001). FBAT perform the testing by a two-step procedure. First the test statistic is defined showing the association between the trait locus and the marker locus. In the second step the distribution of the data of genotypes are tested under the hypothesis. The genotypes of the offspring are treated as random (Rabinowitz and Laird 2000).

Results and Discussion

Linkage mapping. The multipoint linkage map revealed good evidence *RLN3* being mapped on SSC2 in relative position of approximately 23 cM (SW2443 -*RLN3* - SW240) and *GPCR135* being mapped on SSC16 (S0111 - *GPCR135* - S0026). The positions of *RLN3* and *GPCR135* genes found by linkage mapping using CRIMAP are in good agreement with the published assignment to HSA19p13.2, and HSA5p15.1-p14 respectively (Matsumoto et al. 2000, Bathgate et al. 2002). By comparative mapping of pig chromosome to human, we confirmed mapping of *RLN3* and *GPCR135* gene to SSC2 and SSC16 respectively (Meyers et al. 2005).

Expression analysis. The expression study showed *RLN3* as highly expressed in lung, testis and uterus, moderate in spleen, tonsil, lymph nodes, liver, kidney and skin, lower in muscle, heart brain, teat and inverted teat whereas *GPCR135* is moderate expressed in muscle, heart, spleen, kidney and uterus (figure 1). The porcine *RLN3* was expressed in a number of tissues and was similar with *RLN3* expression in mouse. *RLN3* mRNA in mouse is present in several tissues including the brain, thymus, spleen, lung, testis, ovary, and mammary gland and weakly expressed in the heart, liver, epidermis, prostate, and uterus, confirming that relaxin is not just a hormone of pregnancy (Bathgate et al. 2002).

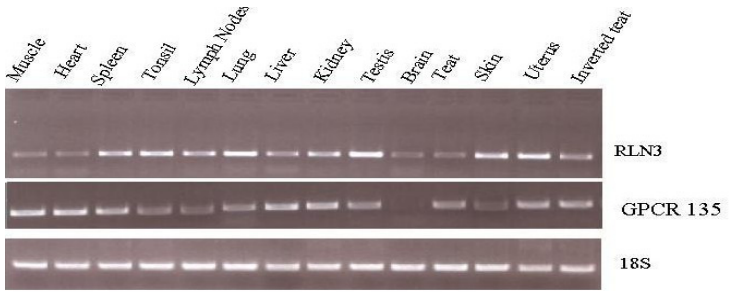

Figure 1. Expression levels of *RLN3* and *GPCR135* mRNA in different porcine tissues (RT-PCR).

SNP screening. Screening for polymorphism revealed two SNP in *RLN3* gene, who are located in intron (C1163T) and second exon (A2338G), respectively. Two alternative alleles could be detected within intron (allele C: 434 bp fragment, allele T: 359 and 75 bp fragments). In a 334-bp fragment within the second exon, two alleles were generated (allele A: 334 bp, and allele G: 217 and 117 bp). Two SNP (C to A) and (C to T), could also be detected in the *GPCR135* gene and genotyped by PCR-RFLP. A 859-bp product was amplified including the alternatives of allele A (350 bp, 276 bp and 202 bp fragment), and allele C (276 bp, 202 bp, 186 bp, and 164 bp). A 198-bp fragment was amplified including allele T (131 and 57 bp), and allele C (75, 57 and 56 bp). The SNP at nucleotide position 2338 (A2338G) of the *RLN3* gene, which mapped to codon 101 of the C chain of second exon, leads to an amino acid change from glutamine (Q) to arginine (R) in the C peptide region of the protein. The prediction for amino acid changes of interest were analysed (http://blocks.fhcrc.org/sift/SIFT.html). For *RLN3* gene the substitution at position 101 from R to Q is predicted to affect protein function with a score of 0.03.

Genotyping and association analysis. The association analysis between each SNP of *RLN3* and *GPCR135* gene with affection on inverted teats defect showed no significant effect in both populations. Haplotypes, constructed using the FBAT program revealed only significance of *RLN3* gene haplotypes.
Allele frequencies of the SNP of the *RLN3* gene were 0.19 for the allele C and 0.81 for the allele T in the animals of the experimental population. For the second SNP the allele frequencies were 0.84 for allele A

and 0.16 for allele G. The frequencies of the haplotypes T-A, C-G, T-G, and C-A were 0.73, 0.13, 0.08 and 0.06, respectively. Only the haplotype T-A was significant associated to the inverted teat defect. The allele C was the most common allele of the first SNP of the *GPCR135* gene in the same population (frequency 0.64). For the second SNP C and T allele frequencies were 0.73 and 0.27 in the experimental population. No significant associations were detected for the *GPCR135* gene in this population.

In the commercial population the allele frequencies of the first SNP of the *RLN3* gene were 0.15 (allel A) and 0.85 (allele T) and for the second SNP 0.74 (allele A) and 0.26 (allele G). The haplotype frequencies were 0.79 (T-A), 0.1 (C-G), 0.08 (C-G) and 0.03 (T-A). For the haplotypes T-A, C-A and T-G a significant association to the inverted teat defect could be detected in the commercial animals. The allele frequencies of the *GPCR135* gene were 0.72 (allele C) and 0.28 (allel A) for the first SNP and 0.8 (allel C) and 0.2 (allele T) for the second SNP.

The inverted teats in *RLN3*-mutated pig could be explained in terms of the absence of connective tissue changes and the lack of smooth muscle relaxation. This is in accordance with relaxin deficient mice which had small size of the nipple and decreased mammary gland epithelial tissue, which are consequences of the changes in the level of circulating relaxin during pregnancy (Zhao et al. 1999). In this study, we could show *RLN3* gene is involved in the development of the inverted teat defect in pig. Expression analysis using Real-Time RT-PCR may give further evidence of the role of member of the relaxin/insulin family for the teat development and the udder defects in pigs. Further investigation will also focus on the influence of both genes to the number of teats in pigs.

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