Analysis of porcine MUC4 gene as a candidate gene for prolificacy QTL on SSC13 in an Iberian × Meishan F2 population

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Abstract

Background: Reproductive traits, such as prolificacy, are of great interest to the pig industry. Better understanding of their genetic architecture should help to increase the efficiency of pig productivity through the implementation of marker assisted selection (MAS) programmes.

Results: The Mucin 4 (MUC4) gene has been evaluated as a candidate gene for a prolificacy QTL described in an Iberian × Meishan (Ib × Me) F2 intercross. For association analyses, two previously described SNPs (DQ124298: g.243A>G and DQ124298:g.344A>G) were genotyped in 347 pigs from the Ib × Me population. QTL for the number of piglets born alive (NBA) and for the total number of piglets born (TNB) were confirmed on SSC13 at positions 44 cM and 51 cM, respectively. The MUC4 gene was successfully located within the confidence intervals of both QTL. Only DQ124298:g.344A>G MUC4 polymorphism was significantly associated with both NBA and TNB (P-value < 0.05) with favourable effects coming from the Meishan origin. MUC4 expression level was determined in F2 sows displaying extreme phenotypes for the number of embryos (NE) at 30-32 days of gestation. Differences in the uterine expression of MUC4 were found between high (NE ≥ 13) and low (NE ≤ 11) prolificacy sows. Overall, MUC4 expression in high prolificacy sows was almost two-fold increased compared with low prolificacy sows.

Conclusions: Our data suggest that MUC4 could play an important role in the establishment of an optimal uterine environment that would increase embryonic survival during pig gestation.

Background

Prolificacy traits have been widely explored during the last decades as a potential tool for increasing efficiency of sow productivity in the pig industry. Genetic improvement programmes have achieved moderate gains in prolificacy related traits owing to their low heritability, late expression in life and sex limitation [1]. Increasing knowledge on the genetic architecture of prolificacy traits would provide new tools to improve the efficiency of genetic selection by implementing marker assisted selection (MAS).

So far a relatively low number of quantitative trait loci (QTL) for prolificacy traits reaching the genome-wide significance level have been identified [2,3]. The most significant QTL affecting the number of piglets born alive (NBA) and the total number of piglets born (TNB) were described by Noguera et al. [3] in the same resource population as used in the present study, an Iberian (Ib) by Meishan (Me) F2 intercross. A number of epistatic QTL were also detected, thus indicating that the genetic architecture of reproductive traits is built as a complex network of interactions throughout the genome. Some of these epistatic QTL were further confirmed and putative candidate interacting genes were identified [4].

Porcine chromosome 13 (SSC13) harbours the most significant QTL for TNB and NBA [3]. The Mucin 4 (MUC4) gene is located within the confidence interval of prolificacy QTL. Mucins are large heterodimeric glycoproteins commonly located on apical surfaces of many wet-surfaced epithelia that play a key role in the lubrication and protection of the uterine mucosa [5-7]. They have been shown to present anti-adhesive and anti-recognition properties which are necessary to protect the endometrium from the
binding and invasion of the trophoectoderm [8,9]. A role of MUC4 has been pointed out in rodents and pigs during pregnancy although its expression during the peri-implantational period varies depending on the type of implantation in each species. In mice and rats, which have an invasive type of implantation, MUC4 expression is downregulated to generate the receptive state for uterine implantation [8-12]. Conversely, in pigs, where a non-invasive epitheliocorial placental attachment takes place, MUC4 is upregulated in the uterus [13]. A protective role has been suggested for MUC4 owing to the fact that it is localized on the endometrium epithelium blocking the access of different substrates to the cell surface [14]. The endometrium is then protected from proteolytic activity of porcine conceptus [13] and from microbial invasion [15] resulting in better uterine conditions for embryo development. In pigs, the disruption of the uterine microenvironment could affect embryo viability which could lead to prenatal mortality rates ranging from 20 to 46% [16]. The improvement of the uterine microenvironment would increase embryonic survival and, in consequence, the number of piglets born alive.

In humans, polymorphisms in the MUC4 nucleotide sequence have been significantly associated with the development of endometriosis and endometriosis related infertility [17]. However, no association with implantation failure has been detected [18]. In livestock species, the genetic association of MUC4 gene variants with reproductive traits has not yet been explored. In pigs, polymorphisms in the MUC4 gene were shown to be in linkage disequilibrium with susceptibility/resistance to Enterotoxigenic Escherichia coli (ETEC) F4ab/ac infection [19].

In the current study, we have examined the porcine MUC4 gene as a functional and positional candidate gene to explain the prolificacy QTL previously identified on SSC13 in the Ib × Me population [3].

Results
Refinement of SSC13 QTL for NBA and TNB
The resulting linkage map for SSC13 in the Ib × Me population was as follows (distance is indicated in centimorgan (cM)): S0076 (0.0) - ITIH3 (18.7) - SWR1008 (27.5) - MUC4 (39.1) - SW398 (55.8) - SW2440 (78.5) - SW769 (91.5). The position of the MUC4 gene is in agreement with the pig genome sequence (Sus scrofa, Ensembl release 64 - September 2011).

A single QTL scan (model 1) was performed on SSC13 for TNB and NBA (Figure 1). As previously described by Noguera et al. [3], two significant QTL for NBA and for TNB were identified at positions 44 cM and 51 cM, respectively (Table 1). Significant additive effects were detected for both QTL which determined an increase of 0.65 ± 0.22 piglets per copy for NBA and 0.51 ± 0.22 for TNB. It is noteworthy that the MUC4 gene was mapped within the confidence interval (CI) of both QTL.

Candidate gene association analyses
Allele frequencies for DQ124298:g.243A>G and DQ124298:g.344A>G SNPs in the Ib × Me population are shown in Table 2. Associations between MUC4 polymorphisms and reproductive traits were tested with a standard animal model (model 2). No significant associations were found for the DQ124298:g.243A>G SNP and the reproductive traits recorded (data not shown). For the DQ124298:g.344A>G SNP, the results supported significant additive effects between this SNP and NBA and TNB traits (P-value < 0.05, Table 1). The additive substitution effect for DQ124298:g.344G SNP was estimated to be 0.74 ± 0.27 for NBA (P-value = 0.006) and 0.57 ± 0.27 for TNB (P-value = 0.037). In both cases, the G allele coming from the Me breed had a favourable effect. Note that DQ124298:g.344A>G SNP had a larger effect on NBA than on TNB (P-value = 0.006 for NBA and P-value = 0.037 for TNB).

The results obtained with association studies must be interpreted with caution due to the fact that the extensive linkage disequilibrium described in F2 crosses can bias the estimations. For this reason, in order to detect possible false positives obtained in association studies with DQ124298:g.344A>G SNP, a marker assisted association test (MAAT) including the QTL effect in the association test [20] was performed with model 3. Results from the
MAAT are summarized in Table 1. The DQ124298:g.344A>G SNP effect on NBA and TNB remained significant when the QTL effect was considered which is in agreement with the association studies analyses. Nevertheless, the significance of the QTL disappeared when the DQ124298:g.344A>G SNP was included in the model. MAAT shows that the DQ124298:g.344A>G SNP genotype explains better the effects on NBA and TNB and confirms the association detected with model 2.

Expression analysis of the porcine MUC4 gene

In order to determine whether MUC4 expression could affect prolificacy related traits, we analysed the uterine expression profile of porcine MUC4 in sows that differed in the number of embryos (NE) at 30-32 days of gestation using qPCR. At this time of gestation, the embryo is already attached to the endometrium and the foetal survival rate will be an indication of the final litter size [1]. For this reason, NE was measured as an estimation of prolificacy. Results showed that mRNA expression levels of MUC4 gene were suggestively greater in high (NE ≥ 13, n = 16, mean relative expression = 7.22) than in low (NE ≤ 11, n = 20, mean relative expression = 3.63) prolificacy sows (P-value = 0.07, Figure 2), reaching almost a two-fold increase in the high prolificacy group.

Discussion

Statistical association between the DQ124298:g.344A>G mutation in the porcine MUC4 gene and prolificacy related traits has been reported. No association was found, however, between the DQ124298:g.243A>G SNP and the prolificacy related traits although this SNP is located 100bp upstream of the DQ124298:g.344A>G mutation. The difference in association analysis detected between both MUC4 SNPs indicates that they are not in linkage disequilibrium. The MUC4 gene successfully mapped within the confidence interval of the SSC13 QTL affecting NBA and TNB traits previously described by Noguera et al. [3] using the same resource population. The favourable effect of DQ124298:g.344A>G SNP was inherited from the Me maternal breed, as expected, since Me is more prolific than Ib. The effect of the DQ124298:g.344A>G MUC4 SNP was stronger for NBA than for TNB suggesting that the MUC4 gene could be related to the embryonic survival in the uterus. This hypothesis is supported by the differential expression of the MUC4 gene found in the uterus at 30-32 days of gestation, where the expression was two-fold higher in high than in low prolificacy sows. Nevertheless, further studies must be performed in other unrelated porcine populations to validate the association which was obtained in this study.

Table 1 Results of QTL analyses, association tests and marker assisted association tests for prolificacy traits

| Trait | Pos. (c.i) | P-QTL a | a_MUC4 (SE) a | Pos. b | P-QTL + MUC4 (DQ124298:g.344A>G) | P-QTL | P-MUC4 (DQ124298:g.344A>G) |
|-------|-----------|---------|-------------|-------|-------------------------------|-------|--------------------------|
| NBA   | 44 (22-77) | 0.003   | 0.65 (0.22) | 0.006 | 0.74 (0.27)                  | 63    | 0.003                    |
| TNB   | 51 (33-74) | 0.021   | 0.51 (0.22) | 0.037 | 0.57 (0.27)                  | 64    | 0.019                    |

1 P-values obtained with the model that includes the QTL and the DQ124298:g.344A>G MUC4 SNP effects; each effect(s) was tested by removing it from the null model; a QTL position (in cM); c.i = confidence interval; b QTL additive effect; SE = standard error; c DQ124298:g.344A>G MUC4 SNP additive effect; d QTL position in cM when corrected by the DQ124298:g.344A>G MUC4 SNP effect.

Table 2 Allelic frequencies of the DQ124298:g.243A>G and DQ124298:g.344A>G MUC4 polymorphisms in the Me × Ib F2 population

|     | DQ124298:g.243 |     | DQ124298:g.344 |
|-----|---------------|-----|---------------|
|     | n  A  G       | n  A  G       |
| F0♀ | Meishan       | 18  0  1       | 18  0  1       |
|     | Iberian       | 3   0.50 0.50  | 3   0.50 0.50  |
| F1  |               | 120 0.32 0.68  | 123 0.18 0.82  |
| F2  |               | 206 0.20 0.80  | 202 0.29 0.71  |

Figure 2 Relative quantification (RQ) of the porcine MUC4 gene in the uterus. MUC4 expression in the uterus was measured in 36 F2 sows that were classified into two groups according to the number of embryos (NE) at the sacrifice day (30-32 days of gestation): low (NE ≤ 11) and high (NE ≥ 13).
MUC4 has been identified as a potential regulator of placenta formation in pigs [21]. In porcine uterine surface epithelium, MUC4 gene expression increases during the first 18 days of gestation [13] whereas in rats a reduction in MUC4 gene expression in the uterus is associated with the period of implantation [12]. Differences in MUC4 expression profiles could be explained by the different placenta types of rodents and pigs. Pigs have a non-invasive type of placenta and MUC4 is thought to play a role in protecting the uterus from erosion by the embryo [13,15]. MUC4 and MUC1 are the major mucin proteins expressed in the endometrial epithelium [22,23]. Human MUC1 and MUC4 present highly polymorphic sites with a variable number of tandem repeats (VNTRs). MUC1 VNTR variants have been related to alterations in both T-antigen presentation and in the local immune response in cancer [17,24,25]. These results suggest that mucins may play a role in the immunological processes that take place during the implantation period essential to ensure the correct establishment of maternal-fetal tolerance [26].

Conclusions
MUC4 polymorphism DQ124298:g.344A>G is associated with litter size in the Ib × Me population and, moreover, MUC4 is differentially expressed regarding the number of embryos in uterus at 30 days of gestation. These results suggest that MUC4 may participate in the establishment of an optimal uterine environment essential for adequate embryo development during the early stages of gestation and increase litter size in pigs.

Methods
Animal material and phenotypic measurements
An F2 population was generated by crossing 3 Ib males from the Guadyerbas line (Dehesón del Encinar, Toledo, Spain) with 18 Me females (Domaine du Magneraud, INRA, France). A total of 8 boars and 97 sows from the F1 generation were mated to obtain the F2 progeny in the Nova Genética S.A experimental farm (Lleida, Spain). All animals were obtained according to the European animal experimentation ethics law approved by the Ethical and Care Committee at IRTA.

Measurements of sow reproduction traits including TNB and NBA were recorded in 255 F2 sows during 4 successive parities. In the fifth parity, sows were slaughtered at 30-32 days of gestation, when the uterus samples were collected and the number of embryos (NE) was recorded.

Genotyping of MUC4 polymorphisms
Two MUC4 polymorphisms described by [19], two A-to-G substitution polymorphisms at positions 243 and 344 of intron 17 (GenBank accession number DQ124298), were genotyped in our Ib × Me population by pyrosequencing [27] in a PSQ HS 96 system (Pyrosequencing AB, Uppsala, Sweden). Two PCR amplifications, one for each SNP, were carried out in a 25 µl total volume that included 1.5 mM of MgCl2, 200 µM of each dNTP, 0.75 U of TaqGold DNA polymerase (Applied Biosystems), 320 nM of each primer (Table 3) and 40 ng of DNA. The thermal profile was 95°C for 10 min, 40 cycles at 94°C for 30 s, 58°C for 45 s and 72°C for 1 min and a final extension step of 15 min at 72°C. A multiplex pyrosequencing reaction was performed with 5 µl of each PCR with the primers described in Table 3.

QTL and association analyses
The linkage map of porcine chromosome 13 (SSC13) was constructed with the Build option of CRIMAP 2.4 software [28]. Overall, seven markers were used: five microsatellites that had been previously described by [3], one SNP at the ITIH3 gene (dbSNP accession number ss315834911) and the DQ124298:g.344A>G MUC4 SNP described by [19].

Three models were used to analyse the Ib × Me F2 population data: (1) a QTL model, (2) an association model and (3) a QTL + association model to perform the marker assisted association test (MAAT) proposed by [20]. First, one dimensional QTL mapping was performed with model 1.

\[
y_{ijk} = H_i + O_j + u_k + p_k + C_a a + e_{ijkl} \quad \text{(model 1)}
\]

where \(y_{ijk}\) was the phenotypic data vector for NBA or TNB; \(H_i\) and \(O_j\) were the fixed effects for year-season and parturition order, respectively; \(u_k\) was the random polygenic effect of each individual; \(p_k\) was the environmental permanent effect of the sow; \(a\) was the QTL additive effect; \(C_a\) was the probability of the individual being homozygous for Ib alleles minus the probability of being homozygous for Me alleles at the QTL position of interest; and \(e_{ijkl}\) was the random residual term. The dominance effect was not included in the model because the likelihood ratio test performed indicated that a model with only additive QTL effect fitted better.

Second, association analyses were performed with the MUC4 SNPs (DQ124298:g.243A>G and DQ124298: g.344A>G) with a standard animal model (model 2).

\[
y_{ijk} = H_i + O_j + u_k + p_k + \sum_{k} \lambda_{ik} d_k + e_{ijkl} \quad \text{(model 2)}
\]

where \(y_{ijk}\) was the vector containing the phenotypic data for NBA and TNB and \(\lambda_{ik}\) was a variable that indicated the number of copies (0, 1, or 2) of the \(k^{th}\) allele presented by each individual.

Finally, a combined QTL + association model (model 3) was used to consider the extensive linkage disequilibrium
present in F2 population. Only the DQ124298:g.344A>G SNP was considered because significant results were obtained in model 2.

\[ \gamma_{ijk} = H_i + O_j + u_k + p_{ik} + C_{ait} + \sum_k \lambda_{ik} d_k + e_{ijkl} \]  
\hspace{1cm} \text{(model 3)}

All analyses were performed with Qxpack software [29]. QTL scans were analysed every cM and nominal P-values were calculated with the maximum likelihood ratio test, assuming a \( \chi^2 \) distribution of the likelihood ratio test with degrees of freedom calculated as the difference between the number of parameters in the alternative and in the null models.

**Expression analysis of the MUC4 gene**

Uterine expression of the *MUC4* gene was measured by reverse transcription quantitative real time PCR (RT-qPCR). Total RNA from uterus samples was extracted by means of the RiboPure™ kit (Ambion, Applied Biosystems). One microgram of total RNA in 40 \( \mu \)l reaction was reverse transcribed with the High Capacity cDNA Transcription Kit (Applied Biosystems). Primers for *MUC4* (Table 3) were designed with Primer Express® 2.0 software (Applied Biosystems, Warrington, UK). *Sus scrofa hypoxanthine phosphoribosyltransferase 1* (HPRT1) was used as a reference gene for normalization [for primer sequences see 30]. qPCR reactions were performed in triplicate in a 20 \( \mu \)l final volume including 2X FastStart SYBR Green Master (Roche), 0.3 \( \mu \)M of each primer and 5 \( \mu \)l of the cDNA diluted twenty times on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Warrington, UK). Thermal conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Dissociation curve analyses were performed in order to detect unspecific amplifications. PCR efficiencies of *MUC4* and *HPRT1* genes were calculated to validate the use of the \( 2^{-\Delta \Delta Ct} \) method [31]. The *MUC4* gene expression level according to the NE at slaughter (30-32 days of pregnancy): low (NE \( \leq 11 \), \( n = 20 \)) and high (NE \( \geq 13 \), \( n = 16 \)). Gene expression data were log10 transformed and analysed by a t-test using the Statistical Analysis System (Statistics, V 9.1.3; SAS Institute, Inc., Cary, NC). The significance threshold was set at \( \alpha < 0.05 \).

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Table 3 List of primer sequences used for typing *MUC4* polymorphisms and quantitative PCR

| Application | SNP/Gene | Primer Sequence (5’→3’) | Length (bp) |
|-------------|----------|--------------------------|-------------|
| Pyrosequencing PCR | DQ124298:g.243A>G | F:*tggtgcgctccccagatta<| 195 |
| | | R:gtggctccctaccccat tat | |
| | | P:gtccccccctccagta | |
| | DQ124298:g.344A>G | F:*tggtccctgtcagcagatg | 258 |
| | | R:ccqaaggtgtaagggaa gacag | |
| | | P:ttggggttggggcag | |
| qPCR | MUC4 | F:atgggcttctccagtgga | 66 |
| | | R:tcgcttcagctggaat | |
| | | P:rtcccacagctcggca | |

* 5’ Biotin labelled, F forward primer, R reverse primer, P pyrosequencing primer.
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