Investigation on Association and Expression of ESR2 as a Candidate Gene for Boar Sperm Quality and Fertility

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Contents

ESR2 is involved in oestrogen-related apoptosis in cell cycle spermatogenesis but their effects have not yet confirmed in pig. Therefore, this study was aimed to investigate the association of ESR2 polymorphism with sperm quality and boar fertility traits and to analyse the ESR2 mRNA and protein expression in boar reproductive tissues. DNA samples from 203 Pietrain (PI) and 100 Pietrain × Hampshire (PIHA) pigs with records of sperm quality [sperm concentration (SCON), motility (MOT), semen volume (VOL), plasma droplet rate (PDR) and abnor-

mal spermatozoa rate (ASR)] and fertility [non-return rate (NRR) and number of piglet born alive (NBA)] traits were available. A SNP in coding region of ESR2 (g.35547A > G) in exon 5 was associated with MOT and PDR in the PI and with SCON, VOL, MOT and PDR in PIHA population. For mRNA and protein expression study, a total of six boars were divided into two groups with group I (G-I) and group II (G-II) where G-I characterized for relatively a better sperm quality according to the mean of two groups. mRNA expression was higher in brain and testis than that in all parts of epididymis. Both qRT-PCR and western blot analysis revealed that the ESR2 gene expression and protein expression were significantly higher in testis collected from G-II compared with that of G-I boars. Moreover, ESR2 protein localization in germ cell, Leydig and Sertoli cells, epithelial cells and spermatozoa was remarkable, which indicated the important role of ESR2 in spermatogenesis process. These results might shed new light on the roles of ESR2 in spermatogenesis as candidate for boar fertility, but still the lack of association across populations should be considered.

Introduction

Oestrogens are classically known to play a major role in female reproduction, but there is now compelling evidence that they may also be involved in the regulation of male reproductive functions. Oestrogens are intimately involved in male fertility, and their function is mediated through binding with the oestrogen receptors 1 and 2 (ESR1 and ESR2) (Couse and Korach 1999). It has been speculated that ESR2 could act as a negative regulatory partner for ESR1 (Weihua et al. 2000). The lack of ESR1 leads to reduced epididymal sperm content, reduced sperm motility and fertilizing ability (Couse and Korach 1999), while the overexpression of ESR2 results in germ cell cycle arrest or apoptosis and infertility (Selva et al. 2004). Aschirn et al. (2005) reported a significant association of ESR2 polymorphism with infertility in human with less sperm concentration, testicular cancer and cryptorchidism. The ESR2 mRNA was highly expressed in the epididymis of adult mouse, rat, dog, cat and monkey (Hess et al. 1997; Saunders et al. 1997; Nie et al. 2002; Zhou et al. 2002). Rago et al. (2007) and Saunder et al. (2001) localize ESR2 protein in spermatozoa within the testis in human and primates, respectively. However, the ESR2 gene has scarcely been investigated as a candidate gene for sperm quality and fertility traits in pigs. In pig, ESR2 is located at the telomeric end on the q-arm of SSC1 (Munoz et al. 2004). In this region, QTL for total sperm per ejaculate and sperm motility in boars (Xing et al. 2008) and QTL for number of nipples and age at puberty in sows are reported (Cassady et al. 2001). Before reaching sexual maturity, a number of germ cells undergo physiological apoptotic death, which has been shown to be controlled by a large number of genes, including the ESR2 (Delbes et al. 2004). When germ cell development is complete, the mature spermatids are released from the Sertoli cells into the tubule lumen and proceed through the excurrent duct system, known as the rete testis, until they enter the epididymis via the efferent ducts. In spermatogenesis, functional male gametes are produced through complex processes in the testis, epididymis and other male reproductive tract (Frungieri et al. 2006). Failure in any of these events leads to disturbances in male fertility. ESR2 is expressed in the cellular type during normal spermatogenesis but its function is still unknown. For the better understanding of ESR2 functions in spermatogenesis in pig, the expression and localization of ESR2 at different parts of reproductive tract including non-reproductive tissues are important. Considering together, it could be speculated that ESR2 might be a functional as well as a positional candidate gene for male reproduction traits in pigs. But to the author’s knowledge, no study was devoted to unravel its association with sperm quality and boar fertility traits, and the functions of ESR2 in boar spermatogenesis within the reproductive tracts by mRNA and protein expression are poorly understood. Therefore, the aims of this research were to study the association of ESR2 with boar sperm quality and fertility traits and to investigate the ESR2 mRNA and protein expressions in sperm and reproductive tissues from boars with divergent phenotype.

Material and Methods

Animals and traits used in association study

Semen samples from Pietrain (PI, n = 203) and Pietrain × Hampshire crossbred (PIHA, n = 100) boars were used for association analysis in this study. These animals were used for AI in commercial pig herds in

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north-western Germany. Details of the populations and phenotypes were described previously by Wimmers et al. (2005), Lin et al. (2006) and Kaewmala et al. (2011). In brief, sperm samples of more than 31,000 ejaculates were repeatedly collected from these boars. Whole ejaculates were obtained from purebred Pietrain and crossbred Pietrain × Hampshire boars aged between 2 and 5 years with an average age of 3.5 years. Sperm quality traits included sperm concentration [SCON (×10^6/ml)], semen volume per ejaculate [VOL (ml)], sperm motility [MOT (%)], plasma droplets rate [PDR (%)] and abnormal spermatozoa rate [ASR (%)] and were obtained from each ejaculate employing light microscopic evaluation according to the guidelines of the World Health Organization. Semen was collected by the vinyl glove hand method twice/week. For each boar, the repeated measurements of sperm quality traits were available. Fertility data [non-return rate data (NRR) at 42 days after insemination (%) and number of piglet born alive (NBA) per litter] of each boar were available as the deviation from the population means within sow breed, parity of sow, farm and season classes as described earlier by Lin et al. (2006) and Kaewmala et al. (2011).

**Genotyping of ESR2 SNP**

As a single nucleotide polymorphism, the arginine (A)-to-guanine (G) transversion of ESR2 at g. 35547A>G in exon 5 reported by Munoz et al. (2007) was further investigated in this study. For PCR amplification, the forward (5′-ctctctggatgatttagc-3′) and reverse (5′-atgctctcttcggta-3′) primer pairs were designed covering exon 5 of porcine ESR2 genomic sequence (GenBank accession No. ENSSSCG00000005632) using the Primer3 tool (Rozen and Skaletsky 2000). Polymerase chain reactions (PCR) were performed in a 20-μl volume containing 100 ng of porcine genomic DNA, 1× PCR buffer (with 1.5 134 m M MgCl2), 0.25 m M of each dNTP, 5 pmol of each primer and 0.1 U of Taq DNA polymerase (GeneCraft). The PCR were performed under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, 30 s at 72°C and final elongation of 10 min at 72°C. After checking the PCR products in 1.5% (w/v) agarose gels, genotyping was carried out following the restriction fragment length polymorphism (RFLP) analysis. The digestion of restriction enzyme FarI (New England BioLabs, Ipswich, MA, USA) was carried out in 10 μl of reaction mixture of each sample and incubated overnight at 65°C. Restriction fragment length polymorphisms of 304 boars were detected by electrophoresis in 3% (w/v) agarose gels.

**Statistical analysis for sperm quality traits**

The association of the ESR2 genotypes with sperm quality and quantity traits was carried out using mixed model (PROC MIXED) in the sas software package (SAS Institute Inc., ver. 9.2, Cary, NC, USA) as described by Kaewmala et al. (2011).

\[ y_{ijkl} = \mu + \text{season}_i + \text{genotype}_j + \text{age}_k + \text{ejaculation}_l + \epsilon_{ijkl} \]  

(Model 1)

where \( y_{ijkl} \) is the sperm quality traits (SCON, VOL, MOT, PDR and ASR); \( \mu \) is the overall population mean; season\( _i \) is the fixed effect of the i-th season \( (i = 1 \text{ through } 8) \); four seasons/year, in total eight seasons within 2 years from January 2000 to December 2001; genotype\( _j \) is the fixed effect of the i-th genotype \( (j = 1, 2 \text{ and } 3) \); age\( _k \) is the effect of boar age (covariable); ejaculation\( _l \) is the permanent environmental effect of the l-th boar (random); and \( \epsilon_{ijkl} \) is the residual error. As ejaculation was recorded as repeated measurement, it was considered as random effect in the statistical model.

The association analysis between ESR2 and the fertility traits was carried out using the following generalized linear model (PROC GLM) in sas (Kaewmala et al. 2011).

\[ y_{ij} = \mu + \text{genotype}_i + \text{year}_j + \epsilon_{ij} \]  

(Model 2)

where \( y_{ij} \) is the boar fertility trait (NRR and NBA); \( \mu \) is the overall population mean; genotype\( _i \) is the fixed effect of the i-th genotype \( (i = 1, 2 \text{ and } 3) \); year\( _j \) is the fixed effect of the j-th boar year of birth \( (j = 1 \text{ through } 3) \); boar born before 1996, in 1996–97 and in 1998–99; and \( \epsilon_{ij} \) is the residual error.

The distribution of the genotype was tested for Hardy–Weinberg equilibrium by chi-square (\( \chi^2 \)) test. Least-square mean values for the ESR2 genotypes were compared by \( t \)-test and p-values, adjusted by the Tukey–Kramer correction.

**Selection of animals for mRNA and protein expression**

The reproductive (testis, head, body and tail of epididymis, vas deferens, bulbourethral gland, vesicular glands and prostate gland) and non-reproductive tissues (brain, muscle and liver) from six breeding boars with divergent phenotypes were collected from the AI station (SuissAG, Sempach, Switzerland) for mRNA and protein study as described earlier by Kaewmala et al. (2011). For differential expression study between reproductive and non-reproductive conditions by reverse transcription PCR (RT-PCR) study, mRNA from all six boars were pooled together according to the tissues. On the other hand, the differential mRNA and protein expression study in different reproductive tissues from two divergent groups of animals was performed by RT-PCR, qRT-PCR and western blot, respectively. For these purposes, the six boars were divided into two groups based on extreme phenotypes (high/low SCON, SMOT and SVOL). Correlation between sperm quality traits was carried out using correlation analysis (PROC CORR) in sas. The SCON (average sperm concentration) was highly negatively \( (r = -0.8) \) correlated with SVOL (average semen volume), whereas SCON was highly positively \( (r = 0.7) \) correlated with SMOT (average sperm motility). Moreover, SVOL was highly negatively \( (r = -0.8) \) correlated with SMOT. Therefore, grouping was made on the basis of SCON, SVOL and SMOT (Table 1). The six boars were selected and equally divided into group I (G-I) with high SCON \( (>262.32 \times 10^6 \text{ ml}) \), high SMOT \( (>76.59 \%) \) and low SVOL \( (<215.24 \text{ ml/ejaculation}) \) and group II (G-II) with low sperm concentration and motility and high sperm volume (Table 1). The differ-
ence between the two groups was calculated using PROC T-Test in SAS. There were differences for SCON (p < 0.05) and for SVOL (p < 0.01) between G-I and G-II, whereas for the SMOT, the difference was not significant (p = 0.12).

### Reverse transcription PCR

Total RNA was isolated using TRI Reagent (Guanidinium thiocyanate-phenol-chloroform extraction) (Sigma-Aldrich, Munich, Germany) from different reproductive and non-reproductive tissues of breeding boars mentioned earlier. RNA was purified using RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was treated using on-column RNase-Free DNase set (Promega, Mannheim, Germany) and quantified spectrophotometrically (ND8000; Nano Drop, Thermo Scientific, Wilmington, DE, USA). Furthermore, RNA integrity was checked by 2% agarose gel electrophoresis. First-strand cDNA were synthesized from individual RNA using Superscript II enzyme (Invitrogen, Darmstadt, Germany).

cDNA amplification was performed by using specific forward and reverse primers (forward: 5'-gacgtttggagcttggttac-3' and reverse: 5'-gatttcacctaatgcctct-3') derived from porcine ESR2 sequence (GenBank accession AF164957). Amplification was performed with an initial heating at 95°C for 5 min followed by 35 cycles of 95°C for 45 s, annealing temperature at 58°C for 1 min and 72°C for 1 min, on the PCR Thermal Cycler (BioRad, Munich, Germany). PCR products were electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide. First-strand cDNA were synthesized from individual RNA using Superscript II enzyme (Invitrogen, Darmstadt, Germany).

### Quantitative real-time PCR

For real-time PCR, total RNA was isolated using TRI reagent (Sigma; Sigma-Aldrich, Munich, Germany) from different reproductive tissues of two divergent groups of animals (G-I and G-II) as described in aforesaid section. cDNA synthesis was carried out as described in the previous section. The same primer pairs used in RT-PCR were also used in qRT-PCR. Ninefold serial dilutions of plasmid DNA were prepared and used as template for the generation of the standard curve. In each run, the 96-well microtitre plate contained each cDNA sample, plasmid standards for the standard curves and no-template control. To ensure the repeatability of the experiments, each plate was run in three replications. qRT-PCR was set up using 2 μl first-strand cDNA template, 7.6 μl deionized H2O, 0.2 μl of upstream and downstream primers and 10 μl 1 × Power SYBR Green I master mix with ROX as reference dye (BioRad). The thermal cycling conditions were 3 min at 94°C followed by 40 cycles of 20 s at 94°C and 1 min at 60°C. Experiments were performed using the ABI prism® 7000 (Applied Biosystems, Carlsbad, CA, USA) qRT-PCR system. The housekeeping gene GAPDH (forward: 5'-acccagactggtggattg-3' and reverse: 5'-aactggctctacctctg-3') derived from porcine sequence (GenBank accession No. AF017079) was used for the data normalization. Glyceraldehyde 3-phosphate dehydrogenase was quantified twice as technical replication and the average was used for the normalization of target gene ESR2. Final results were reported as the relative abundance level after normalizing with mRNA expression level of the housekeeping gene. Differences in ESR2 mRNA expression were analysed with the simple t-test in SAS software (SAS Institute Inc., ver. 9.2). Values of p < 0.05 were considered to indicate statistically significant differences.

### Western blotting

The protein was extracted from different reproductive tissues (testis, head, body and tail of epididymis) from the two divergent groups of breeding boars as used in qRT-PCR. However, for western blot study, proteins from three G-I boars were pooled together and proteins from three G-II boars were pooled together according to the tissues. The proteins extracted from tissues were separated by SDS-PAGE (gradient 4–18%). Subsequently, the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). After blocking in blocking buffer (20 mm Tris pH 7.5, 150 mm NaCl, 0.05% Tween-20 and 1% polyvinylpyrrolidone) at room temperature with secondary antibody, followed by washing with six changes of 0.1% PBST (10 min to time). The horseradish peroxidase-conjugated goat anti-goat IgG (Cat.nr. Sc2020; Santa Cruz) was used as the secondary antibody (diluted 1 : 50 000). The membrane was incubated for 1 h at room temperature with secondary antibody, followed by washing with six changes of 0.1% PBST (10 min to time). The chemiluminescence was detected by using the ECL plus western blotting detection system (Amerham Biosciences) and was visualized by using Kodak BioMax XAR film (Kodak, Stuttgart, Germany). Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control and for normalization. The membrane was stripped by incubation in 2% SDS, 100 mm Tris-HCl and 0.1% β-mercaptoethanol for 30 min at 60°C and reprobed with GAPDH antibody (Cat.nr. Sc20357; Santa Cruz).

### Protein localization by immunofluorescence

Owing to the limitations of fresh samples from G-I and G-II boars, we collected different fresh reproductive
tissues from a healthy breeding boar after slaughtering for protein localization by immunofluorescence (Kaeuw-
malra et al. 2011). Immunofluorescence staining was performed on 8-μm cryostat sections of snap-frozen
tissues. All sections were kept at -80°C for further analysis. To block unspecific staining, sections were
incubated for 30 min at room temperature with 5% bovine serum albumin in PBS (50 mM sodium phos-
phate, pH 7.4; 0.9% NaCl). Sections were incubated overnight at 4°C with the ESR2 goat polyclonal primary antibody (Cat.nr. Sc6822; Santa Cruz) diluted at 1: 50 in PBST followed by six times (10 min to time) washing with PBS. Then, the sections were incubated for 1 h at room temperature with the biotinylated donkey anti-

goat IgG-B conjugated with fluorescein isothiocyanate (FITC)-reactive water-soluble fluorescent dye (Cat nr.
Sc2090; Santa Cruz) (dilution 1 : 200) as a secondary antibody for ESR2 followed by six times (10 min to time)
to washing with PBS. Finally, the samples were counterstained with vectashield mounting medium (Vec-
tor Laboratories, Loerrach, Germany) containing 4,6-diamidino-2-phenyl indole (DAPI) and covered with a
cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss, Oberkochen,
Germany). In case of negative controls, PBS was used instead of the primary antibody.

Results
Association study

The polymorphism of ESR2 at g. 35547A > G in exon 5 was used to genotype the PI and PIHA populations by
PCR–RFLP. The DNA restriction fragments obtained for the ESR2-Fat1 polymorphism were 284, 146 and
28 bp for the AA genotype, 284, 174, 146 and 28 bp for the AG genotype and 284 and 174 bp for the GG
genotype. The genotype and allele frequencies of porcine ESR2 gene calculated are shown in Table 2. Homozy-
gote AA was more frequent, and homozygote GG was rare in both populations. The chi-square test revealed
that the locus of ESR2 was in Hardy–Weinberg equi-

librium in both populations (Table 2). The general descriptions of sperm quality and fertility
traits are shown in Table 3. Means of VOL, MOT and PDR traits in the crossbred PIHA were higher com-
pared with those in the PI populations. The NRR42 was
higher in PIHA compared with PI, while the NBA was
associated with lower MOT and higher PDR than animals having a AA genotype in the PI population (Table 4). In the PIHA population, the animals AG and GG genotypes were associated with lower MOT and higher PDR than the animals having a AA genotype in the PI population (Table 4). This association with
MOT and PDR was consistent in PI and PIHA
populations. The genotype AA is different from AG/GG genotype. The genotypes AG and GG were
associated with lower MOT and higher PDR than animals having AA genotype. The polymorphism g.35547A > G of
ESR2 showed highly additive effect on MOT (p < 0.01) in PI population. The results also indicated
higher additive effect on SCON and VOL (p < 0.05) in PIHA population (Table 4).

ESR2 gene expression was by reverse transcription PCR

ESR2 gene expression was higher in brain and testis, and lower expression was found in the head, body and
tail of epididymis. The mRNA expression of ESR2 was not detectable in accessorios gland (vas deferens, bulbo-
urethral, vesicular and prostate glands), muscle and liver. The RT-PCR result of GADPH showed no
remarkable differences among tissues (Fig. 1).

mRNA and protein expression study in tissues from G-I and G-II boars

The ESR2 mRNA was expressed in testis, body and tail of epididymis from both the G-I and G-II boars but higher expression was found in tests of G-II than in that of G-I boars by RT-PCR (Fig. 2a). These mRNA expression results of RT-PCR appeared to be consistent with the results of the qRT-PCR. The ESR2 mRNA expression was higher in testis of G-II compared with that of G-I boars (p < 0.01), whereas the difference in expression level was not statistically significant in case of head, body and tail of epididymis between G-I and G-II boars (Fig. 2b). ESR2 protein with 56 kDa molecular weight was detected in testis, head, body and tail of

<table>
<thead>
<tr>
<th>Population</th>
<th>Traits</th>
<th>Sample size</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>SCON (10^6/ml)</td>
<td>20 077</td>
<td>3.03</td>
<td>0.94</td>
<td>1</td>
<td>6</td>
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<tr>
<td></td>
<td>VOL (ml)</td>
<td>21 248</td>
<td>237.03</td>
<td>57.32</td>
<td>25</td>
<td>522</td>
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<tr>
<td></td>
<td>MOT (%)</td>
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<tr>
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<td>ASR (%)</td>
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<tr>
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<td>NRR42 (%)</td>
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<td>0</td>
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<td>1.27</td>
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<tr>
<td>PIHA</td>
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<td>0.97</td>
<td>1</td>
<td>6</td>
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<td>546</td>
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<tr>
<td></td>
<td>MOT (%)</td>
<td>76 105</td>
<td>85.46</td>
<td>4.03</td>
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<td>95</td>
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<tr>
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<td>PDR (%)</td>
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<td>4.95</td>
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<td>NRR42 (%)</td>
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<td>NBA (per litter)</td>
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<td>0.04</td>
<td>0.51</td>
<td>-2.97</td>
<td>1.10</td>
</tr>
</tbody>
</table>

ASR, abnormal spermatozoa rate; MOT, motility; PDR, plasma droplet rate, SCON, sperm concentration; VOL, semen volume. aFertility (NRR42, NBA) corrected with factors: parity, farm, season and breed.

Table 2. Genotype, allele frequencies and the chi-square test of the porcine ESR2 gene in different pig populations

<table>
<thead>
<tr>
<th>Breed</th>
<th>Genotype frequency</th>
<th>Allele frequency</th>
<th>Chi-square test</th>
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<tr>
<td></td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
</tr>
<tr>
<td>Pietrain</td>
<td>203</td>
<td>0.89(176)</td>
<td>0.10(20)</td>
</tr>
<tr>
<td>Pietrain ⨯ Hampshire</td>
<td>100</td>
<td>0.79(77)</td>
<td>0.18(18)</td>
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epididymis in both G-I and G-II boars (Fig. 2c). The western blot result showed that the ESR2 protein was higher in testis in G-II compared with G-I boars. This protein expression seemed to be consistent with the results of transcription levels.

Localization of ESR2 protein in boar reproductive tissues by immunofluorescence

Sections of testis, head, body and tail of epididymis were stained through the same optical panel for the cell-surface ESR2 protein expression (Fig. 3). The Leydig and Sertoli cells in testis and epithelial cells in all parts of epididymis showed signals for ESR2 immunoreactivity (Fig. 3a–d). Immunoreactive ESR2 protein was observed as strong staining in germ cell cytoplasm of Sertoli cells and Leydig cells in testis (Fig. 3a). ESR2 protein was expressed in epithelial cells of head (Fig. 3b), body (Fig. 3c) and tail of epididymis (Fig. 3d). In case of spermatogenesis, ESR2 protein was expressed in spermatogonia, primary spermatocyte and spermatid (arrow head) within seminiferous tubules (Fig. 3a). The ESR2 protein was expressed in the spermatooza located within the lumen of the body of epididymis. The ESR2 protein was expressed in the acrosomal cap (arrow) of boar spermatozoa (Fig. 3c).

Discussion

Association of SNP with sperm quality and boar fertility traits

This study revealed an association of ESR2 with sperm quality and fertility traits in boars. The exonic SNP g.35547A>G was found to be significantly associated with sperm motility and plasma droplet rate and suggestively (p = 0.06) associated with number of piglet born alive in PI populations, whereas it was significantly associated with sperm concentration, semen volume, sperm motility and plasma droplet rate in PIHA populations. Sperm motility and plasma droplet rate are consistently associated in both the PI and PIHA populations. It could be seen in both populations that AA genotype contributed significantly to have higher sperm motility and less plasma droplet rate (Table 4). It is important to note that sperm motility and plasma droplet are significantly negatively correlated in our populations. In case of PIHA, genotype AA significantly contributed to higher sperm concentration and lower semen volume (Table 4), which are also in agreement with our correlation results that SVOL and SCON are significantly negatively correlated. In case of ESR2, association has been described in sows by Munoz et al. (2004, 2007) but they did not find any statistically

<table>
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<tr>
<th>Population</th>
<th>Trait</th>
<th>ESR2 genotype (μ ± SE)</th>
<th>Effect (μ ± SE)</th>
<th>Additive</th>
<th>Dominance</th>
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<td>Pietrain (PI)</td>
<td>SCON (10^8/ml)</td>
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<td>VOL (ml)</td>
<td>AA</td>
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<td></td>
<td>MOT (%)</td>
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<td>85.38 ± 0.24</td>
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<td>PDR (%)</td>
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<td>5.04 ± 0.19</td>
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<td>7</td>
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<td>0.58 ± 0.56</td>
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<td>NBA (per litter)</td>
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</tr>
<tr>
<td>Pietrain × Hampshire (PIHA)</td>
<td>SCON (10^8/ml)</td>
<td>AA</td>
<td>3.16 ± 0.07a</td>
<td>2.98 ± 0.15a</td>
<td>2.33 ± 0.33b</td>
</tr>
<tr>
<td></td>
<td>VOL (ml)</td>
<td>AA</td>
<td>265.34 ± 6.84</td>
<td>271.85 ± 13.94</td>
<td>360.03 ± 31.19</td>
</tr>
<tr>
<td></td>
<td>MOT (%)</td>
<td>AA</td>
<td>85.13 ± 0.47a</td>
<td>82.33 ± 0.97b</td>
<td>83.32 ± 2.18b</td>
</tr>
<tr>
<td></td>
<td>PDR (%)</td>
<td>AA</td>
<td>5.43 ± 0.33a</td>
<td>7.58 ± 0.68b</td>
<td>7.54 ± 1.52b</td>
</tr>
<tr>
<td></td>
<td>ASR (%)</td>
<td>AA</td>
<td>6.06 ± 0.40</td>
<td>7.27 ± 0.82</td>
<td>8.84 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>No. of boars</td>
<td>AA</td>
<td>77</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>NRR42 (%)</td>
<td>AA</td>
<td>0.46 ± 0.53</td>
<td>1.86 ± 1.11</td>
<td>3.31 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>NBA (per litter)</td>
<td>AA</td>
<td>-0.01 ± 0.06</td>
<td>-0.09 ± 0.14</td>
<td>0.01 ± 0.30</td>
</tr>
</tbody>
</table>

ASR, abnormal spermatozoa rate; MOT, motility; PDR, plasma droplet rate; SCON, sperm concentration; VOL, semen volume. a,bp < 0.05; c,dp < 0.01; e,fp < 0.005; *p < 0.05; **p < 0.001.

Fig. 1. mRNA expression of ESR2 in reproductive and non-reproductive tissues by reverse transcription PCR
significant association. However, in human, there are two reported polymorphisms of ESR2 at 1082 (G>A) and 1730 (G>A) in exons 5 and 8, respectively, but only the SNP at 1082 (G>A) showed an association with male infertility (Aschim et al. 2005). This finding is almost similar to our study describing that the heterozygote AG genotype has negative effect on sperm quality. Aschim et al. (2005) reported a significantly increased frequency of the ESR2 AG genotype among infertile men, compared with fertile control. Polymorphism in ESR2 at g.35547A>G in exon 5 had effect on sperm quality traits in this study. This SNP was observed in the coding region of the ESR2 gene leading to an amino acid substitution (Met → Val) in the hormone-binding domain, which may be critical for its role as transcription factor (Munoz et al. 2007). Moreover, this alteration involves the replacement of the non-polar amino acid, valine, by the polar sulphur-containing amino acid, methionine, which could modify the secondary and tertiary structures of the protein because of the different ability of these amino acids to form hydrogen and disulphide bonds. The potential deleterious effect of the mutation p.Val317Met located in the ligand-binding domain is suggested by its conserved amino acid position among some mammalian species (Rattus norvegicus, GenBank accession number: Q62986, Mus musculus: O08537 and Bos taurus: Q9XS5B). In human, it has been reported that the ESR2 polymorphism could have a direct effect through changing the nucleotide sequence and thereby the secondary structure of the ESR2 mRNA, possibly leading to changes in mRNA synthesis, splicing, maturation, transport, translation or degradation (Iida and Akashi 2000). These data support the possible biological relevance of this amino acid change, and we found association with sperm quality traits. However, we could not detect any significant association of these SNPs with fertility traits. We found a suggestive association of g.35547A>G ESR2 with number piglets born alive in PI population (p = 0.06) but not in PIHA population. Munoz et al. (2004) reported that SNP in ESR2 are not associated with litter size in Iberian and Chinese-European sows. In addition, sampling additional animals for DNA sequencing may provide detection of other sequence variants in this gene that could be associated with fertility traits. An association study with more individuals should be conducted with this Met/Val substitution.

mRNA and protein expression in boar reproductive tissue

The ESR2 was highly expressed in brain and testis when compared with other tissues, indicating that this gene might have important functions in these tissues. Repro-
Productive processes were maintained by hormone level initiated in the brain (Balthazart and Ball 1997a; Thompson et al. 1998). Enzyme CYP19 catalyses the oestrogen production in brain, and ESR2 is reported to express at the same area at brain where CYP19 (aromatase subfamily 19) is reported to express (Balthazart 1998). It has been reported that testosterone must be transformed into an oestrogen by aromatization to activate the male sexual behaviour (Balthazart and Ball 1997b). Two responses that were known to be oestrogen dependent are the control of aromatase synthesis and the activation of reproductive behaviour (Balthazart 1998).

The expression of ESR2 gene in the reproductive tissues of G-II was higher than that in G-I boars. The protein expression coincided with mRNA expression for ESR2. There were significantly higher ESR2 mRNA and protein expressions in testis from G-II boars than in the testis from G-I boars. The ESR2 tended to be higher in G-II, indicating that higher mRNA and protein expressions might have negative effect on boar sperm quality and fertility. ESR2 is reported to be expressed higher in the semen of infertile males compared with fertile (Bujan et al. 1993). Moreover, the overexpression of ESR2 is reported to cause apoptosis in cell cycle spermatogenesis (Selva et al. 2004). Another study reported that ESR2 is involved in oestrogen-related apoptosis of germ cells, and as a consequence, there is a blockade of germ cell growth during foetal and neonatal life (Delbes et al. 2004). ESR2 plays a role either in regulating the progression of the first meiotic division or in favouring the entrance of primary spermatocytes to an apoptotic pathway (Selva et al. 2004). One of the mechanisms by which ESR2 might regulate cell cycle is through its direct and specific interaction with the cell cycle spindle assembly checkpoint protein, Mad2 (Poelzl et al. 2000). In the spermatogenesis process, the overexpressed ESR2 interacted with Mad2 in meiosis I in mouse leading to a cell cycle arrest or apoptosis in metaphase I (Wassmann et al. 2003). However, ESR2 gene has been expressed in diseased as well in normal testis (Makinen et al. 2001). It has been reported in ESR2 knockout mice (ESR2KO) that ESR2 is important for maintaining the testicular function (Oliveira et al. 2000). Adult ESR2 knockout mice study showed that inactivation of ESR2 affected the cellular composition of the testis (Gould et al. 2007), which may indeed have a direct role in the spermatogenesis. Yet, it is important to note that ESR2 does not play a role alone in spermatogenesis. ESR2 regulation is most likely of extratesticular origin, and a plausible candidate for the regulation of ESR2 in the testis and epididymis would be

![Localization of ESR2 protein in different parts of the boar reproductive tissues.](image)

(a) Immunofluorescence detection of ESR2 in germ cell and in cytoplasm of Sertoli and Leydig cells. Germ cells were stained with ESR2 (arrows), and the nuclei were counterstained with diamidino-2-phenyl indole (DAPI). (b–d) ESR2 protein localization in epithelial cells in the head, body and tail of epididymis. (e) The ESR2 localized in acrosomal cap (arrow) of spermatozoa (arrow head) within the lumen body of epididymis. (e–h) The cell nuclei were counterstained with DAPI. (i–l) Merged images. (m–p) Negative control. Magnification 40x. T, Testis; HE, Head of epididymis; BE, Body of epididymis; TE, Tail of epididymis.
be luteinizing hormone (LH) because a correlation between ESR2 and LHR (luteinizing hormone receptor) concentration is reported in the male tract (Derecka et al. 1999; Guo et al. 2001). LH controls epididymal differentiation, function and sperm maturation through ESR2, and lack of LHR function results in serious disorders in the development of puberty and fertility (Zhang et al. 1997).

**Localization of protein**

The concentration of ESR2 appears to be high in germ cell and in cytoplasm of Sertoli and Leydig cells in testis. A number of studies reported that ESR2 is predominant in germ cells of rodents and humans (van Pelt et al. 1999; Jefferson et al. 2000; Makinen et al. 2001; Zhou et al. 2002). A similar expression pattern of ESR2 described in this study suggests that the locally produced oestrogens in these cells might act through ESR2. Furthermore, there are reports of ESR2 expression in other germ cell types such as spermatogonia (van Pelt et al. 1999; Jefferson et al. 2000; Makinen et al. 2001; Zhou et al. 2002) and elongated spermatids (Rosenfeldt et al. 1998). Our observations are in agreement with the results reported in adult rodents, primate goats, dog and cats (Goyal et al. 1997; Nie et al. 2002; Hess and Carnes 2003). Expression of ESR2 seemed rather homogeneous within and between cell types, and there was a little variation in staining intensity. These data revealed on the protein level were confirmed on the mRNA level, indicating that transcription and translation of ESR2 occur in the positive staining cells. Oestrogen activity of pig testis is unknown, but it has been reported that there is a functional linkage between ESR2 and embryonic growth of pig (Kowalski et al. 2002). In fact, the ESR2 immunostaining pattern in testicular somatic and germ cells as well as in immature and mature gonads suggested that oestrogens might modulate spermatogenesis and testis development via a differential expression of two oestrogen receptor subtypes (Rago et al. 2007).

In this study, the ESR2 immunostaining was found in the epithelial cells from three epididymal regions. The findings of the present study confirmed that ESR2 is expressed in a specific manner in the epididymis and suggest that oestrogens might modulate the epididymal function. Head, body and tail of the epididymis are reported to be involved in morphological and biochemical sperm maturation, in the progression of sperm towards the vas deferens and in its storage (Carpino et al. 2004). We have found ESR2 in the acrosomal cap of pig spermatozoa within the lumen of the body of epididymis. The acrosomal cap is a cellular site closely related to the exocytotic event preceding the oocyte fertilization, and in human, it has been reported that oestradiol is able to influence capacitation and acrosomal reaction of spermatozoa (Aquila et al. 2005). The localization of ESR2 in the acrosomal region implies its involvement in the fertilization process (Solakidi et al. 2005). However, it is important to note that there are more other proteins identified in the post-acrosomal region of sperm, such as equatorin and oscilin, which are important for successful fertilization (Ramalho-Santos et al. 2002; Solakidi et al. 2005).

**Conclusion**

It might suggest that the higher expression of ESR2 might have negative effect on boar sperm quality validated through association study and by profiling of mRNA and protein expression in non-reproductive and reproductive tissues. Therefore, with regard to the association, the transcript and differential expression and protein localization depending on boar sperm quality traits provide experimental evidence for the role of ESR2 in male fertility. However, the results of this study have to be validated in another breed/crossbreed population in order to evaluate its potential in genomic selection.

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**Conflict of interest**

None of the authors have any conflict of interest to declare.

**Author contributions**

AG performed the experiments and wrote the manuscript; MUC partly supervised the work and revised the manuscript; MJU edited the manuscript; KK performed the experiment with AG; DT was responsible for kits and reagents; CP partly supervised the work; ET was responsible for the statistical analysis; CL revised the manuscript; KS was responsible for the whole experiment and supervised the overall work.

**References**


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