The study of polymorphism within the promoter region of the osteopontin (OPN) gene in sows

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Submitted: 2009-06-22 Accepted: 2009-07-25 Published online: 2009-09-25

Key words: **osteopontin; gene polymorphism; reproduction; pig**

Neuroendocrinol Lett 2009; **30**(4): 525–529 **PMID:** 20010486 NEL300409A13

Abstract

OBJECTIVES: The aim of the present study was to find a possible polymorphism in the promoter region of the osteopontin (*OPN)* gene, as a potential mutation region, connected with the transcription factor-binding sites or regulatory sequences and to estimate the expression of this gene in ovaries and oviduct of sows.
MATERIAL AND METHODS: Sixty wbp x pbz sows after the first mating were slaughtered and tissues samples of the ovaries and oviduct were taken. Primer pairs for PCR analysis were designed on the basis of swine osteopontin 5' end sequence driven from GenBank. This study's amplified DNA fragment, spanning 274 bp of the promoter region, was chosen because of its contents of tree specific sites: type II collagen silence sequence (CACCTCC) at -682 (from the transcription initiation site), glucocortIcoid response site (TGTCCT) at -658 and CAAT box -592. This DNA fragment was subjected to a MSSCP analysis.
RESULTS: A different MSSCP pattern was shown which indicates that mutation is located in this region. The samples of different conformers were sequenced and the A → G transitions was identified in two positions -617 and -608. Restriction analysis of the DNA was performed, but unfortunately none of the known restriction enzymes recognized the novel SNPs, which is why the specific primer pairs characteristic for nucleotide A or for nucleotide G were chosen. In the second stage of the presented study the total RNA was extracted from the tissues of the ovaries and oviduct and the complementary DNA (cDNA) was synthesized.
CONCLUSION: The real-time PCR analysis to determined the expression dynamics of the OPN gene in pig tissues was performed.

ORIGINAL ARTICLE

INTRODUCTION

The molecular approach in the study of porcine reproduction is important not only for basic research but also in clinical studies and animal production. For several years many laboratories have tried to find a gene or genetic marker closely related to reproductive properties. Among the numerous genes found in female reproductive tissue, the *SPP1* gene (secreted phosphoprotein 1), also known as osteopontin (*OPN*), is visibly promising in this respect. Osteopontin is an acidic single-chain phosphorylated glycoprotein found in both female and male reproductive tracts**.** This acid protein is a member of the small integrin-binding ligand N-linked glycoprotein family with extra cellular matrix proteins/cytokines which undergo intensive posttranslational modification, including phosphorylation, glycosylation and cleavage, yielding variants of molecular weight ranging from 25 to 75 kDa (Johnson *et al.* 2003).

The *OPN* gene is expressed in many different tissues, including the epithelial cells of the endometrial and the metrial gland cells of the deciduas within the uterus, the placenta, and the invading trophoblast, during the defined window of receptivity of the periimplantation period of pregnancy in many mammalian species (Johnson *et al.* 1999; Nomura *et al.* 1988). The mentioned studies indicate that the OPN gene plays an important role in embryo implantation and placentation. (King *et al.* 2003). In the search for candidate genes critically expressed in the human endometrium during the window of implantation, osteopontin (SPP1) was found to be 17-fold up-regulated in the array study in the receptive phase and the results were confirmed by RT-PCR (Mirkin *et al.* 2005). The up-regulation of this gene during the implantation window was also shown in earlier experiments (Borthwick *et al.* 2003; Carson *et al.* 2002 ; Kao *et al.* 2002). In pigs, the SPP1 mRNA is expressed in the luminal epithelium in the porcine uterus during the early implantation period, inducing the cytoplasmic reorganization and focal adhesions in the uterus and on the conceptus (Garlow *et al.* 2002). It has been also reported that the significant differences in prenatal survivals between the meishan breed and European commercial breeds are mainly attributed to the marked reduction in peri-implantation conceptus loss seen in the meishan breed (Ford 1997; Wilson *et al.* 1999). Even when the uterus size and the ovulation rate of these two breeds are similar, the meishan breed farrows more viable piglets - three to five more per litter (King *et al.* 2003).

A genome scan, employing anonymous DNA markers to identify genetic markers implicated in reproduction process, is currently used to identify quantitative traits loci (QTL). King *et al.* (2003) identified putative QTL, which is associated with litter size and prenatal survival. It is positioned between markers Sw61 and S0178, close to the microsatellite marker in 5' of the SPP1 gene at the distal end of the long arm of chromosome 8 (SSC8). Earlier studies provided evidence that QTL on this chromosome influences several reproductive traits (Braunschweig *et al.* 2001; Cassady *et al.* 2001; Milan *et al.* 1998; Rathje *et al.* 1997; Rohrer *et al.* 1999 ; Wilkie *et al.* 1999). Variation in a microsatellite repeat in 5' end of the SPP1 gene was shown to be associated with the increased litter size in a Meishan x Large White cross (Van der Steen *et al.* 1997). Korwin-Kossakowska *et al.* (2002) reported associations between the presence of a SINE in the SPP1 gene (identified previously by Knoll et al (1999) and litter size of the parities for 519 sows from commercial polish line 990.

Finally, the localization of the SPP1 gene (within the 95% confidence interval) of putative QTL for litter size and parental survival (correlated to birth weight) suggests roles for SPP1 in porcine pregnancy [White *et al.* 2005). The physiological role of OPN, QTL analysis (with regards to chromosome 8) and previous results concerning the association between the OPN gene and reproduction traits suggests that the gene is a strong candidate among the genes associated with female reproduction.

The aim of the present study was to find a polymorphism in the *OPN* gene's promoter region, as a potential mutation region, connected with the transcription factor-binding sites or regulatory sequences and to estimate the expression of this gene in ovaries and oviduct of sows.

MATERIAL AND METHODS

Animals

Sixty wbp x pbz sows from farms co-operating with the Technical and Agricultural University, Bydgoszcz were used. After the first mating they were slaughtered and tissue samples of the ovaries and oviduct were taken. Simultaneous blood samples from all individual animals were collected.

PCR –RFLP Analysis

Primer pairs for PCR analysis were designed using the Primer 3 software available from the Internet (http://www.genome.wi.mit.edu./cgi-bin/primer/primer3 on the basis of swine osteopontin 5' end sequence driven from GenBank [Accession Number M84121].

The genomic DNA was extracted from the blood samples. A polymerase chain reaction was carried out in a 10 µl reaction mixture containing 30 ng of genomic DNA, 10x reaction buffer, 15 mM $MgCl_2$, 250 mM of each dNTP, 25 pmol of each primer and 1.25 unit of polymerase (Promega).

MSCCP analysis

The DNA fragments amplified in this study were subjected to a *(Multi Temperature Single-Strand Conformation Polymorphism)* (MSSCP). A total volume of 10 µl PCR product was mixed (1:1) with a loading buffer (50% formamide, 0.5 M EDTA pH 8.0, 0.05% bromophenol

TGCATGCTTGCTTCTGCCATTGACATAAATTACCTTCTTCTGTCTGACAT
AGCAATTATCAGATTCAT **CACCTCC**AAACCATTCAAAAATCCA**TGTCCT**T
GAAAGGTCTCGGATTTACAATGGTAAATCCGATTTAATA**A**ACATGAAC**G**T
G **A**
GGATTTGAACGTGA**CCAAT**GCCACTTGTCTAAATGACAAGCTGCTGCACA
TTTGGAATCACAAAACAGAGCTTTAGTCGAAATGGAGAGAAGCTGGCA

Figure 1. DNA Sequence of the analyzed fragment of pigs OPN gene.

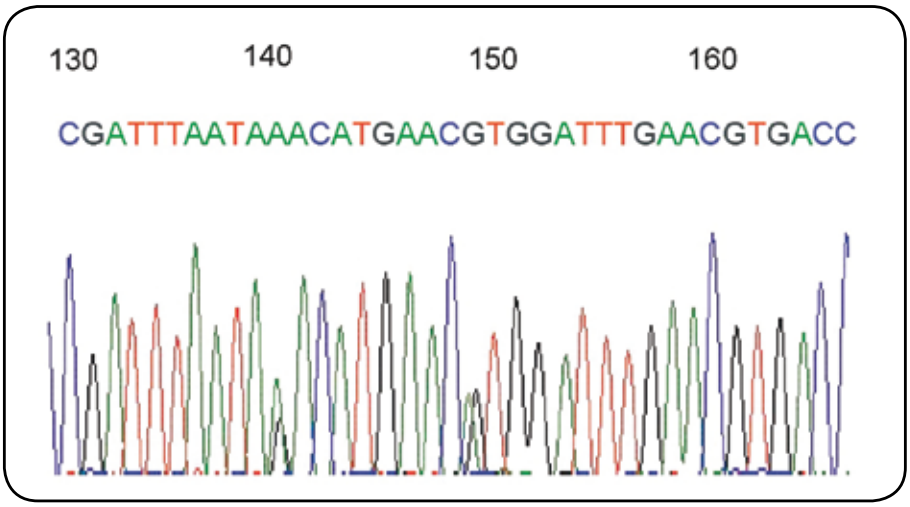

Figure 2. Chromatogram of the analyzed fragment of pigs OPN gene.

Table 1. The specific primer pairs characteristic of A or G nucleotide in analyzed fragment of pig OPN gene promoter

Primer names	Primer sequences
OPN3175a	ATGGATTTGAACGTGACCAA
OPN3175g	GTGGATTTGAACGTGACCAA
OPN4164a	TTATTAAATCGGATTTACCATTGA
OPN4164g	CTATTAAATCGGATTTACCATTGTA

Table 2. Primers used in real-time PCR (included 204 bp sequence of cDNA encoding osteopontin, OPN)

Primer names	Primer sequences
OPN-A	ACGGGAGACCCCAATGAT
OPN-B	GCTGTCCAAGTCAGAAGCCA
β-ACT-A	GGACTTCGAGCAGGAGATGG
β-ACT-B	GCACCGTGTTGGCGTAGAGG
GAPDH-A	ACTCACTCTTCTACCTTTGATGCT
GAPDH-B	TGTTGCTGTAGCCAAATTCA

blue). It was denatured at 95°C for 5 min, chilled on ice and loaded on 8% polyacrylmide gels (29 acrylamide: 1 bis-acrylamide) containing 1xTBE buffer. The gels (180 mm x 130 mm) were run at 300V for 4 h in 2xTBE at the temperature program: 30°C, 18°C and 8°C. The DNA fragment was visualized with the silver-staining technique (*Silver Stain, Kucharczyk Electrophoretical Techniques*) according to the procedure described by the manufacturer.

Sequencing analysis

The PCR products were purified through ultrafiltration using Microcon 100 microconcentrators (Amicon). Purified PCR products (about 280 base pair length) were sequenced with *BigDye® Terminator v1.1 Cycle Sequencing Ready Reaction Kit* (AB) in a GenAmp PCR System 9600 Thermal Cycler (AB) and separated in a DNA sequencer ABI PRISM 3130 (AB) according to the user's manual. The electrophoretic data were analysed by the Sequencing Analysis v.3.0. software (AB). The restriction analysis of the DNA was performed using Webcutter Software (http://rna.lundberg.gu.se/cutter2/).

RNA extraction

The total RNA was extracted from the frozen tissues (ovarian and oviduct tissues) according to the protocol of Chomczyński et al (1987) using *TRIZOL LS reagent* (Gibco) and treated with *DNase* I (in the presence of RNasin) and then subjected to real time PCR analysis.

The concentration of RNA was determined and quality of the RNA was checked in ethidium bromide–stained agarose gel. The complementary DNA (cDNA)

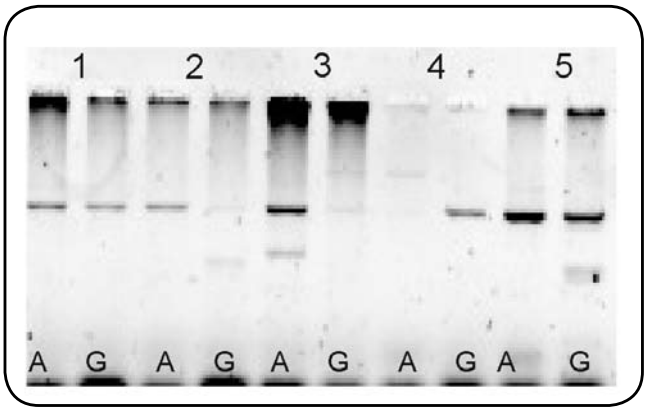

Figure 3. PCR product of the analyzed fragment with using specific primers. Individual 1 - A/G genotype, individual 2 - A/A (no band characteristic for nucleotide G), individual 3 - A/A genotype , individual 4 - G/G (no band characteristic for A nucleotide), individual 5 once again A/G genotype.

was synthesized from a template by using a *Transcriptor First Strand cDNA Synthesis Kit* (Roche). Primers used in real-time PCR included 204 bp sequence (spanning ends of exon 6 and 7) of cDNA encoding osteopontin. The Real-time analysis was perform by using *Power SYBR®Green PCR Master Mix*. The housekeeping genes *β-Actin* and *GAPDH* (glyceraldehydes-3-phosphate dehydrogenase) were used as internal control to normalize expression of the gene of interest.

RESULTS AND DISCUSSION

Only one fragment of size 274 bp appeared to show a different MSSCP *(Multi Temperature Single-Strand Conformation Polymorphism)* pattern**,** which means a mutation occurs in this region. This fragment of the OPN promoter region was chosen because of its contents of three specific sites: type II collagen silence sequence (CACCTCC) at -682 (from the transcription initiation site), glucocortycoid response site (TGTCCT) at -658 and CAAT box at -592 (Zhang *et al.* 1992) (Figure 1). The samples of different conformers were sequenced and the A → G transition was identified in two positions -617 and -608 (Figure 2).

The restriction analysis of the DNA was performed but none of the known restriction enzymes recognized the novel SNPs. Therefore the specific pair of primers, characteristic of A or G nucleotide, was chosen (Table 1). The PCR products were run in a 2% agarose gel stained with ethidium bromide to verify the presence of mutation in *SPP1* (Figure 3).

In the later steps, a relationship will be analyzed between the OPN genotype (based on the novel polymorphism) and the examined reproductive traits in sows**..** Until now a few reproductive traits were noted: four connected with the litter size, two characterized litter weight and interval between consecutive farrowing.

According to the expression analysis, in the present study, sixty sows were slaughtered after the first mating and tissues samples were taken of the ovaries and oviduct. In the first stage the total RNA was extracted to determine its concentration and quality. The complementary DNA (cDNA) was synthesized. Real-time PCR analysis to determine the expression dynamics of the OPN gene in examined tissues was performed. This kind of analysis is fast, easy to use and simultaneously measures gene expression in many different samples for a limited number of genes. In this moment primers for *β-Actin* and *GAPD* were designed and used as internal control (Table 2). We also chose primers used in real-time PCR. These primers included a fragment of cDNA sequence encoding osteopontin (Table 2). The presence of a single and specific 204 bp PCR product was assessed by gel electrophoresis.

Expression of the OPN gene in the female reproductive tract has been evaluated only in a few experiments. Monaco *et al.* (2008a) studied the OPN gene expression in immature and mature swine cumulus cells and oocytes and results showed increased expression of SPP1 in porcine oocytes and cumulus cells. However, maturation significantly decreased the expression of *SPP1* in cumulus cells. The presence of *SPP1* mRNA in oocytes and cumulus cells and the abundance of mRNA before maturation suggests its important role in the period prior to maturation of oocytes. Additional studies are required to determine the specific role of *SPP1* in pig oocyte maturation.

The study by White *et al.* (2005) strongly suggested that SPP1 is a critical component in pregnancy and showed a significant increase of its expression in the uterii of pigs, sheeps, goats, rabbits, rats, mice and humans. Porcine trophoectoderm and luminal epithelium (LE) cells exhibit integrin receptor activation and cytoskeletal reorganization in response to SPP1 binding *in vitro*. Also, Johnson *et al.* (2003) found that uterine OPN expression was visibly detected during the peri-implantation period in humans, pigs and rabbits (Apparo *et al.* 2003). These findings confirm that OPN is an important epithelial protein involved in implementation in several mammalian species with diverse types of implantation and placentation. Recent studies in humans and ewes showed that OPN is abundantly expressed in the glandular epithelium of luteal phase endometrium and this expression is regulated by progesterone (Johnson *et al.* 2000).

It was demonstrated that SPP1 plays an important role in the regulation of porcine early embryo development, *in vitro* and in spite of the fact that the mechanism of its action is still unclear, it is surely involved in the reduction of apoptosis (Hao *et al.* 2008).

Bovine OPN also improves the efficiency of embryo production *in vitro* (Monaco *et al.* 2008b).

Patterns of OPN mRNA expression in different tissues of the reproductive tract of many species suggest that OPN has multiple biological functions in repro-

duction throughout the estrous cycle and during early pregnancy. In support of this hypothesis, it was recently reported that the OPN gene is one of the several putative quantitative trait loci on porcine chromosome 8 associated with reproductive process.

In conclusion, the obtained results describing the mutations are promising in view of a further detailed approach to the identification of favorable variants of the OPN gene genetic marker for an efficient reproduction process.

Acknowledgements

This research was supported by a grant from the Polish Committee for Scientific Research no. N 311 059 32/3421.

The authors gratefully acknowledge Prof. Kazimierz Kochman for critical reading of the manuscript.

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