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POLYMORPHISMS IN DMRT1 CODING AND PROMOTER REGIONS ARE PROBABLY NOT CAUSATIVE FOR SWINE SEX REVERSAL (XX, SRY-NEGATIVE) SYNDROME*

Anna Bratuś[1], Stefan Neuenschwander[2], Agata Piestrzyńska-Kajtoch[1], Marek Świtoński[3], Jolanta Klukowska-Rötzler[4], Ewa Słota[1]

[1]Department of Animal Immuno-and Cytogenetics, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland
[2]Institute of Plant, Animal, Agroecosystem Sciences, Swiss Federal Institute of Technology, Tannenstrasse 1, CH-8092 Zürich, Switzerland
[3]Department of Genetics and Animal Breeding, Poznan University of Life Sciences, Wołyńska 33, 60-637 Poznań, Poland
[4]Institute of Genetics, University of Bern, Bremgartenstrasse 109a, Postfach 8466, 3001 Bern, Switzerland

Abstract

SRY-negative XX sex reversal is an inherited or sporadically occurring disorder, where testis development appears in the absence of the *SRY* gene. Although the molecular background of this intersexuality syndrome in pigs is unknown, it was proposed that familial cases might be inherited as a single autosomal recessive trait. Because *DMRT1* (*Doublesex* and *Mab-3* related transcription factor 1) is an autosomal locus in pig (SSC1q21), shows sexually dimorphic expression in swine gonads and has strong significance in vertebrate testis development, the molecular analysis of this gene was performed in previously reported three intersexes (38,XX, SRY-negative), the progeny of a single boar from a Polish farm. The first two exons encoding functional DM (*double sex* and *mab-3*) domain and the promoter region (the 5'flanking sequence) (altogether 3894 bp) were sequenced and compared with male and female control pigs (n = 16) and with publicly available sequences. Three different polymorphisms were found in the coding region, one Indel type polymorphism (DNA 142_144indelAGC) causing a deletion of an amino acid (protein S47_G48indelS) and two silent SNPs (DNA G432A and G492A). The promoter region seems to be highly polymorphic, since 17 SNPs and 5 indels were detected. However, the sequences of control males and females were concordant with those of the intersexes. These results indicate that *DMRT1* is an unlikely candidate gene for SRY-negative XX sex reversal in pig.

Key words: pig intersexuality, sex reversal syndrome, *DMRT1*, polymorphism

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Although the Y chromosome linked-*SRY* gene was proven to be crucial for mammalian sex determination (Koopman et al., 1991), *SRY* mutations have not been recognized in many reported sexual malformations (reviewed by Sarafoglou and Oster, 2000). One such abnormality is SRY-negative XX sex reversal syndrome observed in individuals with normal female karyotype but developing, in the absence of *SRY* gene, varying amounts of testicular tissue resulting from gonads composed entirely of testis or gonads containing both ovarian and testicular tissues (so-called ovotestis). This intersexuality syndrome has so far been reported in humans (Goodfellow and Lovell-Badge, 1993), goats (Basrur and Kanagawa, 1969; Vaiman et al., 1996), pigs (Thomsen and Poulsen, 1993; Pailhoux et al., 1994; Świtoński et al., 2002), dogs (Meyers-Wallen et al., 1995; Melniczek et al., 1999; Świtoński et al., 2004; Rota et al., 2010), horses (Meyers-Wallen et al., 1997; Buoen et al., 2000), llamas (Drew et al., 1999) and deer (Pajares et al., 2009). In most of the cases it was found to be inherited as a single autosomal or X-linked recessive trait.

Despite the widespread appearance of this hereditary sex reversal syndrome, the causative mutation has been so far described only in humans and goats. Large genomic duplication including the *SOX9* gene (male developmental autosome) was indentified in human XX male in a single report (Huang et al., 1999). More recently, human SRY-negative XX sex reversal was associated with mutations in the *RSPO1* gene (*R-spondin 1*), which seems to be involved in the *WNT* pathway activation (Parma et al., 2006; Tomaselli et al., 2008). In goats, homozygous deletion of a 11,700 bp fragment in chromosome 1 inhibits the expression of neighbouring genes, *FOXL2*, *PFOXic*, *PISRT1* (Pailhoux et al., 2001; Pannetier et al., 2005), which might lead to *SOX9* gene up-regulation and in consequence to male development in XX goats.

Unfortunately, the mutational screening of sex reversal dogs has not yet revealed any causative mutation in canine homologs: *SOX9* (Nowacka et al., 2005) and *RSPO1* (De Lorenzi et al., 2008). In contrast to humans and goats, *PISRT1*, *SOX9* and *RSPO1* were excluded from being causative for dog intersexuality in a large pedigree of American cocker spaniel (Kothapalli et al., 2003; Pujar et al., 2007). These findings altogether might suggest diverse molecular mechanisms underlying development of XX, SRY-negative males in different species.

So far, detailed reports of pig female-to-male sex reversal are available, but no causative mutation has been detected (Thomsen and Poulsen, 1993; Pailhoux et al., 1994; Pailhoux et al., 1997; Świtoński et al., 2002). The isolated cases of this disorder suggest only that maleness development in 38,XX, SRY-negative pigs is caused by a recessive mutation of an unknown autosomal gene.

It was shown that *DMRT1* (*Doublesex* and *mab-3* related transcription factor 1) is an autosomal locus, involved in vertebrate testicular development and is activated in the early embryonic gonadogenesis simultaneously with *SRY* gene expression (XY human embryos; Moniot et al., 2000) or following *SRY* expression (both XX and XY mice embryos; Raymond et al., 1999 b). This gene consists of 5 exons and 2199bp occur in the porcine coding sequence. Despite the missing knowledge of *DMRT1* expression during pig embryogenesis, the recent studies carried out in adult animals have revealed much higher *DMRT1* transcription in testes than

ovaries (Bratuś and Słota, 2009), emphasizing the *DMRT1* involvement in swine male development.

It was also noticed that the *DMRT1* expression level differences might be responsible for abnormal sex phenotype development. Sex reversal in XY women with monosomic deletion of 9p, harbouring *DMRT1*, may be due to haploinsufficiency for the expression of this male regulatory gene (Raymond et al., 1999 a; Muroya et al., 2000). Therefore one can speculate that *DMRT1* over-expression could induce testis development in XX males in absence of the *SRY* gene.

Taking into consideration the probability that *DMRT1* activity alteration is supposed to be causative for sex reversal phenotype, its regulatory sequences and translated region encoding functional DNA binding DM (*double sex* and *mab-3*) domain might affect molecular male developmental pathway by influencing sexual regulators acting both upstream and downstream of *DMRT1*. In this study the promoter region of swine *DMRT1* (whose conservative role in mammalian male differentiation was proved in transgenic mice; Boyer et al., 2002) and first two exons encoding DM domain were sequenced in intersexual pigs in order to evaluate *DMRT1* for candidate gene in swine SRY-negative XX sex reversal syndrome.

Material and methods

Three pig intersexes (38,XX, SRY-negative), all descendants of the same boar, previously described by Świtoński et al. (2002), were investigated in respect of their *DMRT1* genotypes. Sixteen pigs served as controls: Large White (LW): two females (f), two males (m); Polish Landrace (PL): one (f) one (m); Duroc (DU): eight (f), two (m).

Genomic DNA was extracted from peripheral blood using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega, USA).

The fragment of pig *DMRT1* studied is presented in Figure 1. Seven PCR products covering this fragment were amplified by primers designed in PRIMER3 software (http://primer3.sourceforge.net/webif.php) (Table 1). The PCR conditions were as follows: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 45 s, annealing of primers at the temperature indicated in Table 2 for 45 s, elongation at 72°C for 1 min; and final elongation at 72°C for 30 min.

For searching porcine *DMRT1* polymorphisms, both strands of DNA amplicons of intersexes and controls were sequenced with the 3130*xl* Genetic Analyser (Applied Biosystems, USA), using Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), according to the manufacturer's instructions. Sequence analysis was performed with the BioEdit program (version 7.0.8, http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) and blasted with online tools (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) against porcine *DMRT1* reference sequences available in Ensembl (ENSSSCG00000005236) Entrez (NM_214111, AF426435) and *Sus scrofa* genome database assembly 9, published by Sanger Institute (ftp://ftp.sanger.ac.uk/pub/S_scrofa/assemblies/Ensembl_Sscrofa9/).

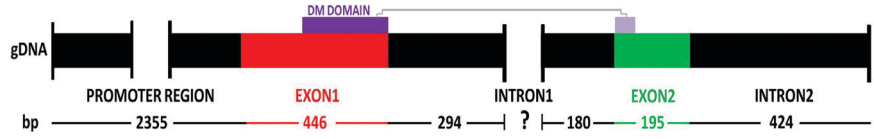

Figure 1. The fragment of swine *DMRT1* gene analysed in the study. Sequence lengths (bp) refer to NCBI and ENSEMBL databases. The length and the positions of DM domain refer both to DMRT1 protein UniProtKB/Swiss-Prot Q9TT01-1 (black violet) and were established by own *in silico* homology studies to human sequence (Accession no. NM_021951; Raymond et al 1999a) (black and light violet)

Table 1. Primers for swine DMRT1 fragments amplification and for sequencing studies

Primer pair	Sequence (5'-3')	Aniling tem., °C	Reference sequence no.	Amplicon	PCR product size, bp
I.	CGTTGTGAGTCCCGACCT	60.5	ENSSSCG00000005236[1]	exon1	728
	CCCCCTGAGCAGAGTAAACC		NM_214111.1[2]	+flanking sequences	
II.	TGGTGGCTGCACATCATAGT	60.5	ENSSSCG00000005236	exon2	811
	TTCCTCACTTCCCAGTGTCC		NM_214111.1[2]	+flanking sequences	
III.	CACAGATTCCTAGCCCCAAA TTCTTGGCCACAACCCTAAG	60	AF426435[2]	promoter region	618
IV.	TCAGGCAGAGAACACTAATGGA CCTTACCTCTAAGGCCAGCA	60	AF426435	promoter region	604
V.	ATGGCCACTACAAGGTCAGG TCTACCTGCTGGCTCCAGAT	60	AF426435	promoter region	948
VI.	TGAGTTTCCAGTGCTTGCAG AGTCGAAGGAAGCCTCTGCT	60	AF426435	promoter region	864
VII.	AACTTGGAGAATGGCGACAC TGTATGCGTCGTCGTTGG	60	AF426435 NM_2141112	promoter region	631

[1]Gene ENSEMBL number accessed in ENSEMBL database (www.ensembl.org).
[2]Accession number accessed in GenBank ((http://www.ncbi.nlm.nih.gov).

Table 2. Pig *DMRT1* gene polymorphisms detected in the study

Polymorphism	Genotypes						Location	SSC1 position, bp[4]
	intersex animals			controls (16 animals)[3]				
	1	2	3	homozygote allele[1]	hetero-zygote	homozygote allele[2]		
359[1]C>G	GG	CG	CG	CC (10)		GG (6)	promoter	231,185,082
390[1]T>C	CC	TC	TC	TT (10)		CC (6)		231,185,051
501[1]C>T	TT	CT	CT	CC (10)		TT (6)		231,184,940
649[1]T>G	GG	TG	TG	TT (10)		GG (6)		231,184,792
738_739[1] indelC	-/-	C/-	C/-	CC (10)	C/- (1)	-/- (5)		231,184,700
743[1]C>T	TT	CT	CT	CC (9)	CT (1)	TT (6)		231,184,694
951[1]G>A	AA	GA	GA	GG (9)	GA (1)	AA (6)		231,184,484
953[1]C>T	TT	CT	CT	CC (9)	CT (1)	TT (6)		231,184,482
1026_1027[1] indelC	-/-	C/-	C/-	CC (9)	C/- (1)	-/- (6)		231,184,410
1375_1376[1] indelAT	-/-	AT/-	AT/-	AT/AT (9)	AT/- (1)	-/- (5)		ND
1381[1]T>A	AA	TA	TA	TT (9)	TA (1)	AA (6)		ND
1383[1]A>T	TT	AT	AT	AA (9)	AT (1)	TT (6)		ND
1384[1]A>T	TT	AT	AT	AA (9)	AT (1)	TT (6)		ND
1390[1]T>A	AA	TA	TA	TT (9)	TA (1)	AA (6)		ND
1392[1]T>A	AA	TA	TA	TT (9)	TA (1)	AA (6)		ND
1519[1]G>A	AA	AG	AG	GG (8)	AG (1)	AA (7)		231,183,912
1520[2]G>A	AA	AG	AG	GG (8)	AG (1)	AA (7)		231,183,911
1675_1676[1] indelT	-/-	T/-	T/-	-/- (6)	T/- (9)	TT (1)		231,183,755
1887[1]C>T	TT	CT	CT	CC (9)	CT (1)	TT (6)		231,183,544
1952_1953[1] indelA	AA	A/-	A/-	-/- (9)	A/- (1)	AA (6)		231,183,480-231,183,479
2077[1]C>T	CC	CC	CC	CC (15)	CT (1)			231,183,355
2432[1]A>G	GG	AG	AG	AA (9)		GG (7)		ND
249_251[2] indel AGC	-/-	-/-	-/-	-/- (7)	AGC/- (1)	AGC/AGC (8)	exon 1	ND
C>A	CC	CC	CC	CC (15)	CA (1)		intron 1	231,182,448
C>T	TT	TT	TT	CC (9)		TT (7)		231,182,321
G>T	TT	TT	TT	GG (9)		TT (7)		231,182,287
539[2]A>G	GG	AG	AG	AA (9)	AG (1)	GG (6)	exon 2	231,177,954
599[2]A>G	GG	AG	AG	AA (9)	AG (1)	GG (6)		231,177,894
T>C	CC	TC	TC	TT (9)	TC (1)	CC (6)	intron 2	231,177,730
A>G	GG	AG	AG	AA (8)	AG (2)	GG (6)		231,177,623
A>G	AA	AA	AA	AA (15)	AG (1)			231,177,513

[1]Positions given according to *DMRT1* promoter region sequence (GeneBank AF426435).
[2]Positions given according to mRNA *DMRT1* sequence (GeneBank NM_214111.1).
[3]Numbers in brackets indicate the numbers of animals showing the same genotype.
[4]Polymorphism positions on SSC1 (database Sus_scrofa.Sscrofa9.53.fa).
ND-No Data available.

Results

Pig *DMRT1* polymorphism in the coding region

Three polymorphisms were detected in the coding region: one indel polymorphism (DNA 249_251indelAGC; protein S47_G48indelS) in exon 1 and two silent SNPs (DNA G539A; protein R144 and DNA G599A; protein P164 – within the composition bias of Poly-Proline region P163_P168) in exon 2; all of which were outside of the functional DM domain (DNA C306_G446, protein R67_Q113) (nucleotide and amino acid positions refer to Gene Bank NM_214111.1 and UniProtKB/Swiss-Prot Q9TT01-1, respectively). Moreover, three SNPs were found in intron 1 and three other SNPs in intron 2 (detected in PCR products amplified with primer pair I and II). All polymorphisms in exons and in their flanking sequences have been observed in both affected and control animals (Table 2).

Pig *DMRT1* polymorphism in the promoter region

The *DMRT1* promoter region (AF426435, Boyer et al., 2002), appeared to be very polymorphic, since 17 SNPs and 5 indels were detected in 19 investigated animals (Table 2). The observed polymorphisms were concordant when comparing the intersex animals with control male and female pigs.

It was noticed that all investigated *DMRT1* genotypes in both coding and promoter regions were the same for two intersexes, designated as 1 and 2 (Table 2).

Discussion

Pig intersexuality has been well described compared to other species. Its frequency ranges from 0.1 to 0.6 % (however, it may reach 20% in isolated herds) and was recognized mostly in adults by breeders because of abnormal external genitalia or at slaughter through the discovery of one or two abdominal testes/ovotestes. Genetically, pig intersexes, in more than 90% of cases, have female karyotype (38,XX) without any Y chromosome sequences, including *SRY* (Pailhoux et al., 1994; Pailhoux et al., 1997). Three affected animals, investigated in the present study, exhibited this type of intersexuality (SRY-negative XX sex reversal syndrome), having female external genitals with an enlarged clitoris, bilateral testes without spermatogenesis activity and cytogenetically and PCR proved female chromosome constitution (38, XX) with no *SRY* (Świtoński et al., 2002).

Intersex animals tested in this study appeared in the progeny of a single unaffected ancestor (38,XY male) (Świtoński et al., 2002). Previously, Pailhoux et al. (1994) also described the pig pedigree that included five intersex animals whose parents did not exhibit sex-reversed phenotype. An autosomal recessive mutation was proposed to be responsible for intersexuality development in the family of tested animals after careful calculation of intersexes frequency among all the offspring of the sire, the carrier of the putative mutation (Świtoński, 2002). Such mode of inheritance was also suggested in other familial cases of SRY-negative XX sex reversal syndrome

in pigs (Pailhoux et al., 1997), goats, horses and dogs (reviewed by Vaiman and Pailhoux, 2000).

Because of the inheritance of this syndrome, the causative allele could be spread in the population, which can lead to some economic losses resulting from sterility, growth reduction and carcasses discrimination because of sexual odour coming from testes (Pailhoux et al., 1994).

Unlike intersexuality in goats and dogs, no extensive studies locating the mutation was carried out in pigs. Two different affected sex determination events might underline the development of XX, SRY-negative males. The first one could be the loss-of-function mutation in the autosomal locus of female regulator (whose wild type inhibits male specific genes) and in consequence leads to masculinization of XX animals (proposed by Pailhoux et al., 1994). The second one might be the gain-of function mutation in the autosomal locus of male regulator that could induce testis development in the absence of the *SRY* gene (as in mouse model, Vidal et al., 2001).

Because of the autosomal status of *DMRT1* (in pigs SSC1q21, Bratuś and Słota, 2009), its well documented involvement in mammalian testis development (Raymond et al., 2000) and its dimorphic expression pattern in swine gonads with higher levels in testes than ovaries (Bratuś and Słota, 2009), the screening for *DMRT1* gain-of-function mutation was carried out in three pig intersexes tested. However, the DNA sequencing data presented in this study (Table 2) showed that neither the translated fragment of the gene encoding functional DM domain nor promoter region of swine *DMRT1* differ in sex reversed pigs. However, the investigated fragment of the gene has been shown to be highly polymorphic: 3 polymorphisms in coding region (but outside of functional DM domain) and 22 polymorphisms in promoter region (in its 5' flanking sequence). This is quite surprising in the light of the previous studies which have presented both structurally and functionally conservation of pig *DMRT1* promoter among mammals (Boyer et al., 2002). Moreover it was noticed that DNA differences among animals were rather due to breed than sexual status. *Sus scrofa* genome database assembly 9 was derived from a single Duroc male called TJ Tabasco, the intersexes were crossbreeds with the major share of White Large, the controls were of different breeds, including Duroc, Large White and Polish Landrace. No data concerning sequence source are available in the pig *DMRT1* promoter (AF426435) and cDNA (NM_214111.1) sequences submitted to GeneBank.

Moreover, the Blast results showed that the promoter region seems to be not yet completely determined: eight detected polymorphisms (Table 2) have shown no assignments to *Sus scrofa* genome database assembly 9. Additionally, one fragment around 250 bp length (556_805nt, according to AF426435) was unexpectedly detected to be a highly repetitive sequence throughout the porcine genome (blast results obtained in BLASTN 2.2.21 software (Altschul et al., 1997) and performed against *Sus scrofa* genome database assembly 9).

We cannot definitely exclude *DMRT1* as a candidate gene for swine XX sex reversal syndrome, as the complete genomic sequence of the gene was not investigated. Similarly, the latest study of Pujar et al. (2007) carried out in the family of American cocker spaniel with 10 sex reversal offspring has also not definitely excluded the

canine *DMRT1* to be responsible for dog intersexuality (XX, *SRY* negative). However, no significant *DMRT1* point mutation has been found in 17 individuals with *SRY*-negative XX sex reversal (Raymond et al., 1999 a). On the other hand, the extensive human, goat and dog studies showed that the molecular background of this sexual disorder can differ between species.

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ANNA BRATUŚ, STEFAN NEUENSCHWANDER, AGATA PIESTRZYŃSKA-KAJTOCH, MAREK ŚWITOŃSKI, JOLANTA KLUKOWSKA-RÖTZLER, EWA SŁOTA

Polimorfizm regionów kodującego i promotorowego genu *DMRT1* prawdopodobnie nie odpowiada za zespół odwróconej płci (XX, brak genu *SRY*) u świń

STRESZCZENIE

Zespół odwróconej płci u osobników o żeńskim kariotypie, pozbawionych genu *SRY* (XX, brak *SRY*) i posiadających jądra (o różnym stopniu rozwoju) może pojawiać się spontanicznie lub być dziedziczony. U świni domowej, mimo że do tej pory nie poznano molekularnego podłoża tego typu interseksualizmu, wykazano, że jego dziedziczna forma może być powodowana recesywną mutacją w autosomalnym locus nieznanego genu. Ze względu na fakt, że *DMRT1* (*Doublesex* and *mab-3* related transcription factor 1) jest genem autosomalnym (SSC1) o dobrze udokumentowanej roli w determinacji płci męskiej u kręgowców i wykazującym zróżnicowaną ekspresję w gonadach u świni, w pracy podjęto badania molekularne nad tym genem u trzech osobników z zespołem odwróconej płci (38,XX, brak genu *SRY*), wcześniej opisanych jako potomstwo tego samego knura, urodzone na pojedynczej farmie w Polsce. Zarówno dwa pierwsze eksony genu, kodujące funkcjonalną domenę DM (*double sex* and *mab-3*), jak i region promotora -5'sekwencja flankująca (razem 3894 bp) poddano analizie sekwencjonowania u chorych osobników i porównano otrzymane sekwencje do świń kontrolnych obydwu płci oraz do sekwencji dostępnych w bazach danych. Wykazano 3 różne polimorfizmy w sekwencji kodującej genu: polimorfizm typu indel ((DNA 142_144indelAGC), powodujący delecję pojedynczego aminokwasu (białko S47_G48indelS) oraz dwie ciche mutacje punktowe (DNA G432A and G492A). Promotor *DMRT1* u świni okazał się wysoce polimorficzny, gdyż znaleziono w jego obszarze 17 polimorfizmów typu SNP oraz 5 polimorfizmów typu Indel. Niestety wszystkie zidentyfikowane polimorfizmy u interseksualnych świń pojawiły się także w sekwencjach DNA zwierząt kontrolnych, czyniąc gen *DMRT1* mało prawdopodobnym locus odpowiedzialnym za rozwój zespołu odwróconej płci u świń.