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Polymorphism of the *CTNNB1* and *FOXL2* Genes is not Associated with Canine XX Testicular/Ovotesticular Disorder of Sex Development*

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78,XX testicular or ovotesticular disorder of sex development (DSD) is the most common sex anomaly in dogs, but its molecular background remains unknown. It was hypothesized that the causative mutation may reside in canine chromosome 23 (CFA23), where two genes playing a pivotal role in ovarian development (*CTNNB1* and *FOXL2)* are located. The aim of our study was to search for polymorphism in both candidate genes in 15 DSD dogs (78,XX and a lack of the *SRY* gene) and 29 normal females. Altogether, 7 novel polymorphic variants were identified: 5 SNPs in *CTNNB1* and 2 indels in the *FOXL2* gene. The distribution of the identified variants was similar in the DSD and control dogs. Therefore, we concluded that the conducted research did not prove an association between these polymorphisms and canine testicular or ovotesticular XX DSD.

Key words: Dog, disorder of sex development, intersexuality, sex reversal, beta-catenin, *CTNNB1, FOXL2*.

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Testicular or ovotesticular disorder of sex development in dogs with a female karyotype and a lack of the *SRY* gene (78,XX DSD) is the most common sex abnormality in dogs, to date identified in more than 30 breeds (MEYERS-WALLEN 2012; BUIJTELS *et al.* 2012; SALAMON *et al.* 2014). The affected dogs, apart from the presence of testes or ovotestes, usually also have virilized external genitalia, e.g. an enlarged clitoris containing a bone, a prepuce-like vulva, and an extended anogenital distance (MEYERS-WALLEN 2012). This DSD is most probably inherited as a sex–limited autosomal recessive trait, as shown for an American Cocker Spaniel family (MEYERS-WALLEN & PATTERSON 1988) and it is supposed to be likely also in other breeds (POTH *et al.* 2010). Until recently the search for a causative mutation did not bring conclusive results (MEYERS-WALLEN 2012). However, a very recent report showed that in some cases a duplication of a large fragment, consisting of the *SOX9* [*SRY (sex determining region Y)-box 9*] gene, was observed (ROSSI *et al.* 2014). On the other hand, our recent study (SALAMON *et al.* 2014) excluded the earlier suggestion (SWITONSKI *et al.* 2011) that the mutation may be localized in the pericentromeric region of chromosome 23 (CFA23). Interestingly, two important genes for ovarian development are located on this chromosome: *CTNNB1* (*Catenin [Cadherin-Associated Protein], Beta 1*) and *FOXL2* (*forkhead box L2*).

The *CTNNB1* gene is activated by two signaling molecules: RSPO1 (R-spondin 1) and WNT4 (wingless-type MMTV integration site family, member 4), but on the other hand the encoded protein promotes expression of other genes in the ovarian determination pathway, i.e. *FOXL2*, *BMP2* and *FST* (MAATOUK *et al.* 2008; NEF & VASSALLI 2009; MEYERS-WALLEN 2009). Moreover, it has an anti-testis function, by preventing binding of SF-1 to a regulatory region of *SOX9* (MAATOUK *et al.* 2008). It is also known that mutations of main activators of the *CTNNB1* gene (*RSPO1* and *WNT4*)

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lead to 46,XX testicular DSD in humans (MANDEL *et al.* 2008; PARMA *et al.* 2006).

The *FOXL2* gene, encoding a forkhead/winged helix transcription factor, belongs to a large gene family involved in developmental processes in vertebrates (for a review see KAUFMANN & KNÖCHEL 1996). The FOXL2 protein is synthesized in somatic cells of the ovary (SCHMIDT *et al.* 2004) and is known as the earliest marker of ovarian differentiation in mammals (COCQUET *et al.* 2002). XX mice lacking the *FOXL2* and *WNT4* genes demonstrate complete sex reversal (OTTOLENGHI *et al.* 2007). Moreover, deletion of an 11.7 kb sequence in a regulatory region localized ~300 kb upstream from *FOXL2* leads to the development of the polled intersex syndrome (PIS) in goats with a female karyotype (60,XX) and a lack of the *SRY* gene (PAILHOUX *et al.* 2001; BOULANGER *et al.* 2014). The aim of this study was to conduct a comparative analysis of the distribution of novel polymorphisms in the coding sequence of the *CTNNB1* and *FOXL2* genes in XX testicular or ovotesticular DSD dogs and in normal female dogs. This analysis facilitates verification of the hypothesis whether the mutations of these candidate genes are responsible or associated with testicular or ovotesticular 78,XX DSD in dogs.

Material and Methods

The Department of Genetics and Animal Breeding, Poznan University of Life Sciences is certified to carry out laboratory diagnostics for veterinary and animal breeding purposes. Biological material for the genetic analyses described below was submitted by veterinary surgeons and sampled from dogs during standard veterinary examination/procedures.

Three groups of dogs were analyzed. The first one contained 15 dogs with an enlarged clitoris, a female karyotype (78,XX) and absence of the *SRY* and *ZFY* genes. Among them 4 dogs (1 Bernese Mountain Dog, 2 Cocker Spaniels and 1 Leonberger) presented ovotesticular and 3 (2 American Staffordshire Terriers and 1 French Bulldog) testicular XX DSD. In the other 8 dogs (1 Kerry Blue Terrier, 2 German Shepherd dogs, 2 American Staffordshire Terriers, 1 Miniature Pinscher, 1 Yorkshire Terrier and 1 Tibetan Terrier) the histology of their gonads was unknown and thus they were classified as ”XX DSD dogs of unknown cause”. Two other groups contained 29 unaffected females: 15 of the same or similar breeds represented in the DSD group (control #1) and 14 representing breeds in which XX DSD was not reported so far (control #2). All animals were previously described (for more details see SALAMON *et al*. 2014).

Genomic DNA was extracted from blood with the use of the Blood Mini Isolation Kit (A&A Biotechnology, Poland). Thirteen primer pairs were designed based on canine *CTNNB1* and *FOXL2* (GenBank, NC_006605.3) using the Primer 3 tool (http://primer3.ut.ee/). Details on PCR (polymerase chain reaction) products (sequence, annealing temperature, size) are given in Table 1. Each PCR mix included approximately 50 mM DNA, 0.25 mM of each primer, 0.125 of each dNTPs and 1 unit of Taq Polymerase (EURx, Poland). For optimization the primers covering the first exon of the *FOXL2* Jump Start Red Taq DNA Polymerase (Sigma Aldrich, Germany) and Hot Start Taq DNA Polymerase (Qiagen, Germany) were also used. The amplification was conducted in a Biometra T-Gradient thermocycler (Germany) under the following conditions: initial denaturation at 94ºC for 5 min, 34 cycles of 94ºC for 40 s (denaturation), 54.6ºC to 64.3ºC for 40 s (temperature of primer annealing, specific for the analyzed fragment – Table 1), 72ºC for 40 s (extension) and the final extension at 72ºC for 10 min. Screening for polymorphism in all groups was performed by direct sequencing of obtained amplicons with the use of the BigDye® Sequencing Kit and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems; USA).

The distribution of polymorphic variants in the studied groups was analyzed with the use of the odd ratio (OR) test (HAINES & PERICAK-VANCE 1998).

Results

The entire coding sequence of the canine *CTNNB1* gene consists of 14 exons which range in length from 13 to 339 bp. The 5’ and 3’ untranslated regions (UTR) are 48 and 1081 bp long, respectively (GenBank, NC_00660.3). Sequence analysis of the whole coding region, entire 5’UTR, 669 bp of 3’UTR and exon/intron junctions of the *CTNNB1* (4592 bp) in dogs revealed 5 single nucleotide polymorphisms (SNPs): 1 missense mutation in exon 13, 3 silent mutations in exons 3, 4, 10, and 1 SNP in intron 11 (Table 2).

The canine *FOXL2* gene spans 2792 bp and contains 2 exons (729 and 231 bp), 1 intron, 5’UTR (96 bp) and 3’UTR (497 bp) (GenBank, NC_00660.3). Our analysis of the entire second exon, intron/exon junction and 415 bp of 3’UTR of *FOXL2* (altogether 736 bp) revealed 2 insertions in intron and 3’UTR (Table 2). Unfortunately, we did not obtain PCR amplicons for exon 1, although several primer pairs were tested (data not shown).

All SNPs localized in the coding sequence of *CTNNB1* (c.351T>C, c.663T>G, c.1728C>T,

Table 1

PCR primers and conditions applied for sequence analysis of the *CTNNB1* and *FOXL2* genes

Gene	Primer sequence	Annealing temperature	PCR product length	Localization
CTNNB1	F: ACTAAAAGAGGTTCAGCAGTGG R: GGAGGAGTGAGCAGAAAATGG	61°C	401 bp	5'UTR exon 1
	F: GGAATGGCTACCCAAGGTTT R: GCATTCACCTGAATTTGTGCT	64.3°C	838 bp	exon 2 and intronic flanking regions
	F: TCAGGTGAATGCTGAATTATGG R: AGGCTCCTTGAGAGTTTCCTTT	58.9°C	362 bp	exon 3 and intronic flanking regions
	F: CTCTCAAGGAGCCTCAGAATG R: TGCAAGAGTCCACAGAAGGA	61°C	722 bp	exons 4 and 5 and intronic flanking regions
	F: TGGGTTGGTAATGTGGCTTT R: GTGATCTTGGCTGCAAACTG	58.9°C	391 bp	exon 6 and intronic flanking regions
	F: TGAGCAACAGCCTAACAAATGA R: AGAAGCTGAACAAGAGTCCCA	61°C	374 bp	exon 7 and intronic flanking region
	F: AGGTGGAATGCAGGCTTTAG R: GGAAGATGGAGGGAACCAAT	61°C	1035 bp	part of exon 7, exon 8 and 9, introns 7 and 8
	F: TCTGAGGGAATCTTGGGTGT R: CTGCACAAACAATGGAATGG	56.8°C	248 bp	exon 10 and intronic flanking regions
	F: TGGGAATGTTTGCACCATAA R: CTCACAGCGGCTGCTAAAGT	54.6°C	568 bp	exons: 11 and 12, and intronic flanking regions
	F: ACTTCCCCAAACCTGTTCCT R: GCCACTACTCTCCTGCCAAC	58.9°C	530 bp	exon 13 and intronic flanking regions
	F: ACACTGCCGTTGAGGTTACA R: CGACCAAAAAGGACCAGAAC	58.9°C	784 bp	exon 14 and part of of 3'UTR
FOXL2	F: GTTTGCTCCACGTAGCCTCT R: AGTGATCTCCCGGCACTCT	58.8°C	556 bp	exon 2 and intronic flanking regions
	F: AAATTTCCCCGGATCTTCC R: GCTGCTGGACAAACTTAGGC	57°C	387 bp	Part of 3'UTR

Table 2

Frequencies of polymorphic variants in XX DSD and unaffected dogs

Gene	Polymorphism		Testicular or Ovotesticular XX DSD (n=7)		*SRY*-negative XX DSD with unknown histology of gonads (n=8)		DSD together (n=15)		Control #1 (n=15)		Control #2 (n=14)		Total Control (n=29)	
	nucleotide*	amino acids (gene region)	'1'	'2'	'1'	'2'	'1'	'2'	'1'	'2'	'1'	'2'	'1'	'2'
CTNNB1	c.351T[1]>C	p.Ala117Ala (exon 3)	1.0	0	1.0	0	1.0	0	0.9	0.1	0.9	0.1	0.9	0.1
	c.663T[1]>G	p.Leu221Leu (exon 4)	1.0	0	1.0	0	1.0	0	1.0	0	0.93	0.07	0.96	0.04
	c.1728C[1]>T	p.Ala576Ala (exon 10)	0.86	0.14	0.94	0.06	0.9	0.1	0.93	0.07	0.93	0.07	0.93	0.07
	c.1953-16T[1]>A	– (intron 11)	0.57	0.43	0.56	0.44	0.57	0.43	0.57	0.43	0.64	0.36	0.6	0.4
	c.2092C[1]>A	p.Leu698Ile (exon 13)	1.0	0	0.87	0.13	0.93	0.07	1.0	0	1.0	0	1.0	0
FOXL2	c.729-5_729-4/insC[1]	– (intron 1)	0.14	0.86	0.31	0.69	0.23	0.77	0.23	0.77	0.21	0.79	0.22	0.78
	c.*427_428insTGTT[1]	– (3'UTR)	0.64	0.36	0.94	0.06	0.8	0.2	0.63	0.37	0.75	0.25	0.69	0.31

* – nucleotide position according to GenBank, NC_006605.3

[1] – allele description, e.g.: '1' it is T at polymorphic site c.351T>C and '2' – it is C at this site.

c.2092CA) were rare in the studied groups. Only one intronic SNP (c.1953-16TA) had a minor allele frequency (MAF) above 0.2. In *FOXL2* we identified two insertions: c.729-5_729-4insC (intron) and c.*427_428insTGTT (3'UTR). All genotypes were observed in the studied groups and MAFs were >0.2 (when DSD were considered jointly).

The distribution of the identified polymorphic variants was similar in XX DSD dogs and the two control groups, as shown by the odd ratio test. Therefore, we concluded that the conducted research did not prove an association between the polymorphisms and testicular or ovotesticular XX DSD in dogs.

Discussion

In this study we searched for polymorphism in the coding sequence of the canine *CTNNB1* and *FOXL2* genes in DSD dogs and unaffected females. Altogether, 5 polymorphic sites were identified in the 4592 bp sequence (approx. 1/918 bp) of the *CTNNB1* and 2 sites in 736 bp (1/368 bp) of *FOXL2*. To our knowledge all of them are newly identified in the dog. The length of human and canine *CTNNB1* coding sequences is equal and comprises 2343 bp (GenBank, NM_001098209 and NM_001137652). The human *FOXL2* coding sequence (NM_023067) is longer than that of the dog (XM_003433109) – 1131 bp and 957 bp, respectively. According to dbSNP (NCBI), altogether 55 (23 missense and 32 synonymous; approx. 1/42 bp) and 20 (14 missense and 6 synonymous; approx. 1/56 bp) polymorphisms in the human *CTNNB1* and *FOXL2* coding sequences were identified, respectively. In comparison, our results revealed only 4 polymorphisms in the canine *CTNNB1* coding sequence (approx. 1/585 bp) and none in *FOXL2*. Of course, these results may be biased by the limited number of analyzed animals. However, similar analyses of other canine genes involved in sex determination also revealed a low number of polymorphic variants in coding regions, for example 1/394 bp in *RSPO1* (DE LORENZI *et al.* 2008) and a lack of polymorphic sites in 1539 bp of the *SOX9* coding sequence (NOWACKA *et al.* 2005).

Studies on mammalian *CTNNB1* and *FOXL2* polymorphism are scarce, especially in relation to DSD. In humans, a loss of *FOXL2* function leads to the blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), an autosomal defect characterized by eyelid abnormalities and premature ovarian failure (POF) (CRISPONI *et al.* 2001). Our study is the first report on polymorphism of these genes in dogs.

Testicular or ovotesticular XX DSD was also described in other mammals, including humans (XIAO *et al.* 2013), goats (PAILHOUX *et al.* 2001), horses (TORRES *et al.* 2013), pigs (SWITONSKI *et al.* 2002) and roe deer (PAJARES *et al.* 2009). In two animal species the causative mutations were identified. A deletion of 11.7 kb in the regulatory region localized ~300 kp upstream from *FOXL2* is responsible for this disorder in goats (PAILHOUX *et al.* 2001; BOULANGER *et al.* 2014). In DSD roe deer, analysis revealed three copies of the *SOX9* gene (duplication breakpoints: 890 bp on the 5' side and >1.5 kb on the 3' side of the gene) (KROPATSCH *et al*. 2013). Moreover, in XX testicular DSD pigs a GWAS (genome wide association study) approach indicated that the causative mutation resides in a region harboring the *SOX9* gene (ROUSSEAU *et al*. 2013).

In studies on the molecular background of the canine XX DSD several candidate genes were analyzed, including *RSPO1*, *PISRT1*, *WT1*, *DMRT1*, *GATA4*, *FOG2*, *LHX1*, *SF1*, *SOX9* and *LHX9* (for a review see MEYERS-WALLEN 2012), but no causative mutation was identified. Very recently ROSSI *et al.* (2014) reported that among 7 DSD dogs, in 2 a heterozygous duplication of the *SOX9* gene was identified. One DSD dog was diagnosed as ovotesticular and the other as the testicular DSD. The duplicated region spanned 577 kb, including the *SOX9* gene (3124 bp) and the 5'- and 3'-flanking sequences. Unfortunately, the authors did not show segregation of the duplication in families from which the affected dogs originated. Taking the above into consideration, it can be assumed that this type of DSD is not caused by a single mutation in dogs or the observed variability of the *SOX9* gene copy number is not responsible.

Molecular knowledge on human testicular/ovotesticular XX DSD is most advanced. Mutations of the *RSPO1* gene were described by PARMA *et al.* (2006) and TOMIZUKA *et al.* (2008). Several reports showed that duplication or triplication of long sequences (above 70 kb) in an upstream (approx. 500 kb) regulatory region of the *SOX9* gene is responsible for this disorder (XIAO *et al.* 2013; COX *et al.* 2011; VETRO *et al.* 2011), but this is not a common cause of this type of DSD in humans (TEMEL & CANGUL 2013). Furthermore, some duplications and one microdeletion of the X-linked *SOX3* gene in 46,XX DSD human patients were also identified (for a review see MOALEM *et al.* 2012).

The importance of *SOX9* region duplication for abnormal sex development indicates that further studies should be focused on the region harboring this gene. However, it should be mentioned that no point mutation causing DSD was found in the coding sequence of the *SOX9* gene (NOWACKA *et al.*

2005). New molecular techniques, including next generation sequencing (NGS) and multiplex ligation-dependent probe amplification (MLPA), offer a great opportunity to improve the standard of searching for the molecular background of this disorder.

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