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Genome-wide association study and heritability estimate for ectopic ureters in Entlebucher mountain dogs

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Abstract

An ectopic ureter is a congenital anomaly which may lead to urinary incontinence and without a surgical intervention even to end-stage kidney disease. A genetic component contributes to the development of this anomaly in Entlebucher mountain dogs (EMD). However, its nature remains unclear. Using the Illumina CanineHD bead chip, a case-control genome-wide association study was performed to identify SNPs associated with the trait. Six loci on canine chromosomes 3, 17, 27 and 30 were identified with 16 significantly associated SNPs. There was no single outstanding SNP associated with the phenotype and the association signals were not close to known genes involved in human congenital anomalies of the kidney or lower urinary tract. Additional research will be necessary to elucidate the potential role of the associated genes in the development of ectopic ureters in the EMD breed.

Keywords: GWAS, urinary tract development, *Canis lupus familiaris*, dog, CAKUT, CALUT, Entlebucher mountain dog

In dogs, an ectopic ureter (EU) is a rare congenital anomaly in which one or both ureteral orifices are not located at the anatomically correct position at the vesicular trigone of the bladder (Osborne *et al.* 1995). Instead, the ureteral openings can be found in the bladder neck, the urethra or even in the genital system (Dean 1988; Owen 1973). The most common clinical sign of affected dogs is urinary incontinence (Fossum 1997). However, many dogs, especially males, which show a higher prevalence for EU than females in the Entlebucher mountain dog (EMD) (Fritsche *et al*. 2014), are continent for years and may show incontinence only at an advanced age (Holt & Moore 1995; Reichler *et al.* 2012). Affected dogs are predisposed for ascending infections of the urinary tract including pyelonephritis; they may have or develop hydroureter and hydronephrosis, as well as changes of kidney size or distorted internal kidney architecture (Holt & Moore 1995; Lamb & Gregory 1998; Niesterok 2016). This may result in fatal end-stage kidney disease if no surgery is performed. The EMD is one of the dog breeds that have a higher risk for EU (Fritsche *et al.* 2014). Out of 552 classified EMD nearly half showed intravesicular ectopia, i.e. 130 females and 132 males. One fifth, i.e. 25 females and 84 males, had at least one extravesicular ectopic termination. Urinary incontinence was a complaint in 3% and 27% of them. Hydronephrosis and/or hydroureter was noticed by ultrasonography in 1% and 14% of the intravesicular and extravesicular cases, respectively. In one third of them this was an incidental finding (Fritsche *et al*. 2012). The breed predisposition indicates a genetic background, however exaggerated breeding restrictions to reduce the risk of clinically affected dogs in the offspring are of concern, as the EMD has already a high average inbreeding coefficient around 40% (Schrack *et al.* 2017). Therefore, selection based on genotypes would be desirable, but first attempts to associate five suitable candidate genes, selected from mouse studies (Uetani & Bouchard 2009), to EU in EMD were unsuccessful (North et al. 2009). In humans, congenital anomalies of the kidney and urinary tract (CAKUT) are a genetically heterogeneous group of developmental disorders (syndromic and non-syndromic) with highly varying phenotypes.

Even though single gene mutations were shown to cause renal anomalies in mice, the elucidation of CAKUT cases in humans remains difficult (Nicolaou *et al*. 2016). Genetic heterogeneity, modifier genes, allelic variation in gene expression, epigenetic effects, complex modes of inheritance and environmental effects may hamper the clarification of the genetic basis of sporadic and familial CAKUT (Yosypiv 2012).

Using complex segregation analysis, our group previously showed a genetic background for EU in EMD and the possible involvement of a major gene for the EU-3 phenotype (Fritsche *et al.* 2014), while the evaluation of an X-linked mode of inheritance, which could explain the observed male predominance for extravesicular ectopia was not successful (Fritsche *et al.* 2014). The goal of the present study was to re-estimate the heritability for EU-3 and identify genetic variants associated with EU and candidate genes within chromosomal regions of such variants. Ureteric openings were determined for 1421 EMDs born between 1996 and 2016 and registered by official national kennel clubs. The majority of dogs were between six and twelve months old when ultrasonographical screening was performed by authorized private veterinarians and university clinics. This screening method was previously validated by comparison with dissection (Balogh *et al*. 2015) and already used to elucidate the mode of inheritance of EU by multivariate mixed logistic regression (Fritsche *et al.* 2014). All EU diagnoses were finally assessed by one researcher in order to avoid a bias through the first examiner. The dogs were classified based on the more caudally placed ureteral opening into three phenotype classes, namely EU-1 (both ureters in the correct anatomical position, i.e. a distance between the more caudal ureteral opening and the vesicourethal junction of at least 1.8 cm (Balogh *et al*. 2015; Rozear & Tidwell 2003)), EU-2 (a distance of the more caudal ureteral opening and the vesicoureteral junction between 1.1 and 0.1 cm) and EU-3 (at least one ureteral opening located extravesically in the urethra or genital tract). Dogs which had not been examined or for which no conclusive diagnosis was attained, were classified as EU-0. Pedigree information for the dogs was available through an in-house

EMD database, merging all EMDs into one single family. We re-estimated the narrow sense heritability (h^2) of EU-3 under a threshold-liability model (Lee *et al.* 2011) using phenotypes of 98 cases (73 males and 25 females) and 151 EU-1 controls (32 males and 119 females) and pedigree information of 4522 dogs. The analysis included sex as a fixed effect. EU-3 was found to be heritable, with estimates of 0.713 and 0.960 in the 0-1 risk and liability scales, respectively. These findings led us to hypothesize that a major risk locus may underlie EU-3, which could be presumably detected through a genome-wide association study (GWAS).

For the GWAS, genomic DNA was extracted from EDTA blood samples of 381 EMDs with phenotype EU-1 (n=218), EU-2 (n=28) or EU-3 (n=135). Genotyping was performed using the Illumina® CanineHD assay (Illumina Inc., San Diego CA, USA) at GeneSeek (part of Neogen Corporation in Lincoln, USA). This chip contains approximately 173000 single nucleotide polymorphism (SNP) markers distributed throughout the genome with an average density of 70 markers per million base pairs, allowing for a robust within-breed association analysis.

Due to the high selective pressure and inbreeding levels in EMD, our GWAS analysis included dominance and autozygosity effects, apart from additive marker effects (see supplementary material). The family-wise error rate was controlled by adopting a LD-corrected Bonferroni significance level of 0.05/K, where K is the effective number of independent markers. The approach was similar to the simple method (Gao *et al.*, 2008), except that K was estimated from the ratio between the total number of markers and the average number of tag-partners per marker ($r2 > 0.3$), instead of the eigenvalues required to explain 99.5% of the variance in the genotype matrix. After scanning all autosomes (Fig. 1), we found six genome-wide significant loci (p-value < 9.65×10^{-5}), which are presented in Table 1.

Significant markers were observed in five regions on canine chromosomes (CFA) 3, 17, 27

and 30. On CFA 3, SNP BICF2P957732 is located near *MCTP2,* which encodes a transmembrane protein with Ca^{++} binding domains involved in signal transduction or membrane trafficking (Shin *et al.* 2005). Lalani and coworkers (2013) found gross heart anomalies in *Xenopus* embryos associated with morpholino knockdown of *MCTP2*. The SNP BICF2P527992 is in an intron of the IQ motif containing GTPase activating protein 1 gene (*IQGAP1*) and presented a significant dominance effect on the investigated trait. *IQGAP1* is an interesting functional candidate gene because it is involved in the regulation of the beta-catenin/GATA3 pathway in *Xenopus* embryos (Goto *et al.* 2013). The beta-catenin/GATA3 pathway is involved in the formation of the ureteric bud, and loss of function of *GATA3* leads to ectopic ureteric bud formation and severe urogenital abnormalities (Grote *et al.* 2008). However, *Iqgap1*-null mice did not show any observable phenotype (Li *et al.* 2000). There is no clear functional candidate gene for the association signal on CFA 17, however, the underlying effects were due to autozygosity in this locus. The most intriguing association signals are on CFA 27. There is one signal at ~1 Mb overlapping with the *HOXC* gene cluster. *HOX* genes are obvious functional candidate genes for phenotypes that involve potential defects in development (Mallo & Alonso 2013). The other association signal at ~23 Mb on CFA 27 is located in an extremely gene poor region upstream of the *SOX5* gene, which encodes another transcription factor involved in development. Coding variants may lead to Lamb-Shaffer syndrome in humans, a neurodevelopmental disorder, sometimes seen in combination with variable skeletal abnormalities (Nesbitt *et al*. 2015) but extremely rarely with urogenital malformations (Lee *et al.* 2013). Therefore, it remains unclear whether *SOX5* is the causative gene underlying this association signal. As the region of this association signal is extremely gene poor, it might have important regulatory functions that are not necessarily restricted to *SOX5* (Ovcharenko *et al.* 2005). The association signal on CFA 30 is located between the *SMAD6* and *SMAD3* genes, encoding again important transcription factors of the TGF-beta signaling pathway that also have a role in development

(Macias *et al.* 2015). While our GWAS failed to pick up any candidate genes known from human studies, it is remarkable that 3 of the 5 putative association signals fall near the genes for developmental transcription factors. As the p-values only just exceed the significance threshold, the findings need to be regarded with caution and the associations should be validated in a larger set of animals. In addition, nine genes which were previously shown to be involved in congenital anomalies of the lower urinary tract (CALUT) in humans (reviewed in Rasouly & Lu 2013) were analysed by comparing the genomes of 296 dogs of different breeds and eight EMD genomes with either EU-1 or EU-3 phenotypes. We did not find any protein-changing variants in these candidate genes (not shown).

Despite the high heritability found for ectopic ureters the association study failed to come up with a single strong association signal. There are at least two possible explanations for such a finding: (1) Despite the high heritability it is possible that many genetic risk loci with small effects are involved in the formation of EU. (2) It also cannot be excluded that a genetic variant, which predisposes the dogs for the formation of ectopic ureters, is fixed in the EMD population and therefore not detectable by the genome-wide association study.
In the latter scenario, modifier genes may still modulate the phenotype despite a unique underlying ectopic ureter genotype, similar to humans (Yosypiv 2012). This assumption is supported by the high average inbreeding coefficient of extant EMDs (Schrack *et al.* 2017), as well as by the much lower prevalence of ectopic ureters in the related Appenzeller mountain dog breed (Bitterli 2011). However, the high heritability seems to contradict this hypothesis. Even if we failed to identify strong candidates known from human studies, the signals seem to be quite clear for the relatively small number of animals, supporting an oligogenic inheritance. However, before using some of those SNPs for marker-assisted selection of breeding EMDs, further research is needed to support this data.

Competing interests

The authors declare that they do not have any competing interests.

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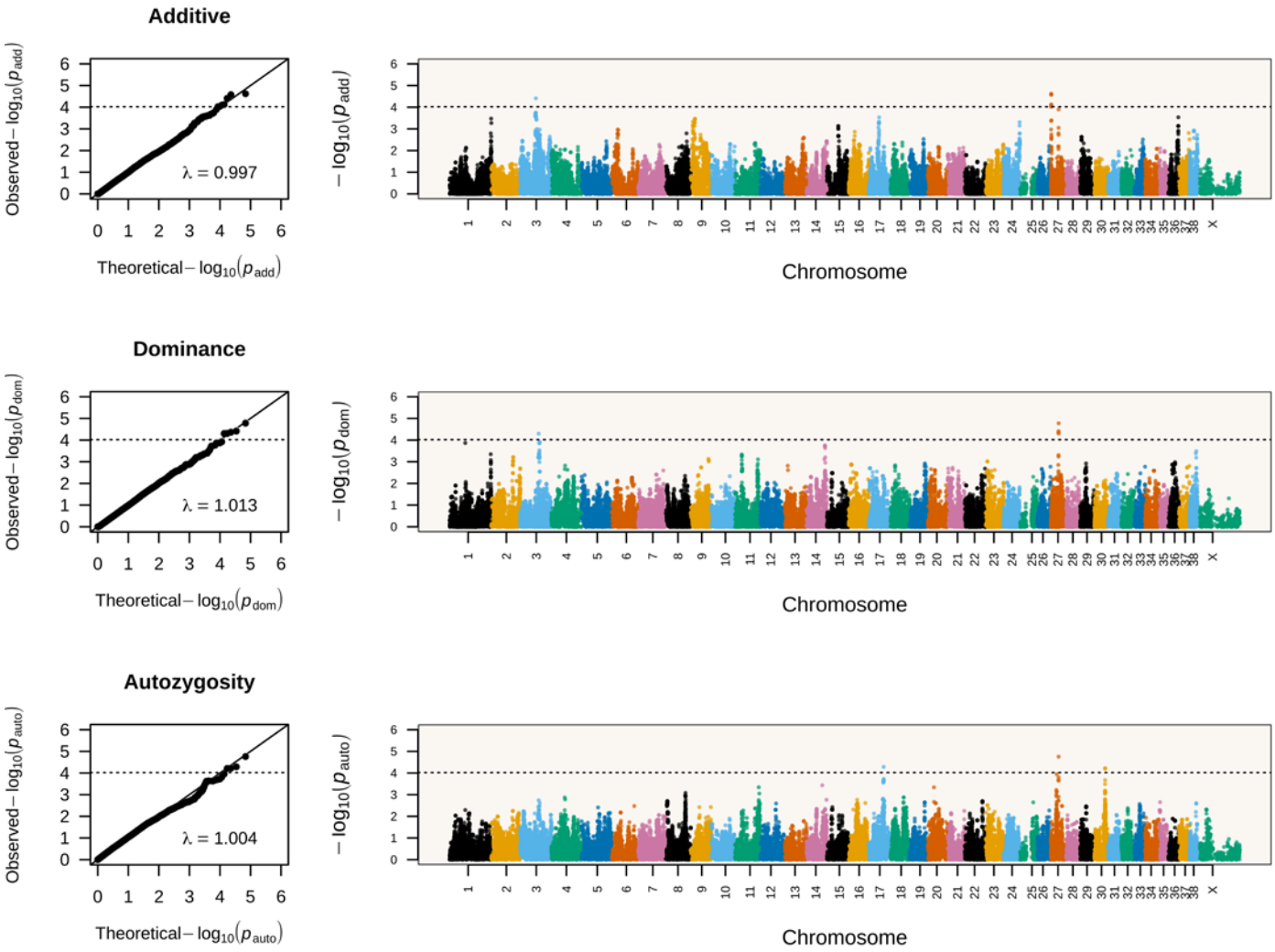

Figure 1 | Quantile-quantile and Manhattan plots for additive (add), dominance (dom) and autozygosity (auto) effects. The dashed horizontal line corresponds to the LD-adjusted Bonferroni threshold (p = 9.65 x 10^{-5}).

Table 1. Summary statistics for significant markers associated with ectopic ureters in Entlebucher mountain dogs

CFA[1]	Position[2]	SNP[3]	Position[3]	[4]Alleles	[5]♂ control	[6]♂ case	[7]♀ control	[8]♀ case	[9]Inheritance model	[10]P-value	[11]nearest gene	[12]Distance
3	45655143	BICF2P957732	45655143	A/C	23/9/0	39/27/6	95/22/2	9/13/3	Additive	3.89E-005	MCTP2	187,272
3	53778402	BICF2P527992	53778402	T/C	13/16/3	43/23/7	57/56/6	18/5/2	Dominance	5.05E-005	IQGAP1	0
17	43412582	BICF2P1360326	43412582	C/T	29/3/0	57/15/0	89/29/1	21/3/0	Autozygosity	5.23E-005	CTNNA2	59,764
27	1007730	TIGRP2P347803_rs8943392	1007730	T/C	20/12/0	65/8/0	95/23/1	24/1/0	Additive	9.38E-005	NEF2	0
27	1168218	BICF2S22927985	1168218	G/A	21/11/0	67/6/0	97/21/1	24/1/0	Additive	9.21E-005	HOXC4	41,292
27	1411816	BICF2G630137589	1411816	G/A	13/17/2	54/19/0	73/42/4	19/6/0	Additive	2.63E-005	HOXC13	88,346
27	1415865	BICF2G630137593	1415865	T/C	13/17/2	54/19/0	73/42/4	19/6/0	Additive	2.63E-005	HOXC13	109,704
27	1428628	BICF2G630137612	1428628	G/A	14/16/2	54/19/0	74/41/4	19/6/0	Additive	7.91E-005	HOXC13	12,763
27	1433130	BICF2G630137624	1433130	C/T	13/16/3	52/21/0	71/44/4	19/6/0	Additive	2.38E-005	HOXC13	17,265
27	1437735	BICF2G630137629	1437735	G/A	14/16/2	53/19/0	74/41/4	19/6/0	Additive	7.47E-005	HOXC13	21,870
27	22783765	BICF2G630149192	22783765	C/T	19/12/1	45/25/2	70/46/3	16/5/4	Dominance	4.39E-005	SOX5	73,896
27	22929398	BICF2P1108722	22929398	A/G	18/13/1	43/28/2	70/46/3	16/5/4	Dominance	3.88E-005	lncRNA	0
27	22994047	BICF2P830285	22994047	T/G	18/13/1	41/30/2	69/46/4	16/5/4	Dominance	5.00E-005	lncRNA	0
27	23114194	BICF2P595351	23114194	T/C	18/13/1	40/31/2	69/44/6	16/5/4	Dominance	1.67E-005	lncRNA	0
30	31140902	BICF2P906072	31140902	C/T	22/10/0	46/23/3	78/37/4	16/8/1	Autozygosity	6.06E-005	SMAD3	105,411
30	31236521	BICF2P664860	31236521	A/G	22/10/0	46/24/3	78/37/4	16/8/1	Autozygosity	6.23E-005	SMAD3	9,792

[1] Canine chromosome, [2]SNP position in the corresponding chromosome (CanFam3.1 genome version) Annotation Release 103, [3]SNPs in the Illumina® CanineHD bead chip, [4]minor allele/major allele, [5-8]SNP genotype distribution, [9]gene effect, [10]P-value, [11]gene symbol of the nearest gene of the reported SNP, [12]Distance in bp between SNP and nearest gene (0=SNP within gene).