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SHORT COMMUNICATION

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Genome-wide association analysis for lethal brachycephalic-like facial dysmorphia in Labrador Retrievers

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Summary

A GWAS was performed for inborn X-linked facial dysmorphia with severe growth retardation in Labrador Retrievers. This lethal condition was mapped on the X chromosome at 17–21 Mb and supported by eight SNPs in complete LD. Dams of affected male puppies were heterozygous for the significantly associated SNPs and male affected puppies carried the associated alleles hemizygously. In the near vicinity to the associated region, *RPS6KA3* was identified as a candidate gene causing facial dysmorphia in humans and mice known as Coffin–Lowry syndrome. Haplotype analysis showed significant association with the phenotypes of all 18 animals under study. This haplotype was validated through normal male progeny from a dam with the not-associated haplotype on both X chromosomes but male affected full-sibs with the associated haplotype.

Keywords association, *Canis lupus familiaris*, facial dysmorphia, *RPS6KA3*, X chromosome

In four litters sired by four different sires, seven male Labrador Retriever puppies were born with a lethal brachycephalic-like facial dysmorphia accompanied by severe growth retardation (Dierks *et al.* 2017). Each dam gave birth to two litters and the dams were a daughter–mother pair. The affected male puppies showed doming of the forehead, shortening of the upper jaws and to a lesser degree of the lower jaws and an abnormally opened fontanelle (Fig. S1). Clinical signs were reduced body weight, inspiratory dyspnea and reduced suckle reflexes. Two of the puppies displayed a severe dorsoventral flattening of the thorax. This condition was not compatible with survival and the puppies had to be euthanized at an age of 3 weeks. An X-linked recessive inheritance was most likely due to the segregation pattern of cases in the pedigree (Fig. 1). Oculo-skeletal dysplasia and swimmer puppy syndrome were ruled out through necropsy findings and a gene test for oculo-skeletal dysplasia (Dierks *et al.* 2017).

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The objective of the present study was to perform a GWAS for this lethal X-linked facial dysmorphia in Labrador Retrievers.

EDTA–blood or tissue samples were available for 18 Labrador Retrievers from this pedigree with this brachycephalic syndrome. Tissue samples were obtained from five euthanized affected male puppies and other 13 unaffected female and male family members.

Genotyping was performed on the canine high-density BeadChip (Illumina) with 173 662 SNPs. PLINK version 1.09 was used for quality control with minor allele frequencies greater than 0.05, genotyping rate greater than 90% and less than 10% missing genotypes per individual, leaving 98 217 SNPs for the GWAS. The canine X chromosome (CFAX) was equidistantly covered by 2215 informative SNPs. The positions of the SNPs were according to the CanFam2.0 genome assembly, and the corresponding SNP positions in CanFam3.1 were determined using NCBI remap (https://www.ncbi.nlm.nih.gov/genome/tools/remap). The ALLELE procedure of SAS/GENETICS, version 9.4 (SAS Institute), was used to calculate polymorphism information content, heterozygosity, allelic diversity, allele and genotype frequencies, and chi-square tests for Hardy–Weinberg equilibrium. The GWAS was performed using a mixed linear model with TASSEL, version 3.0.88 (Bradbury *et al.* 2007). We applied a Bonferroni correction using the

MULTIPLE TEST procedure of SAS, version 9.4, to determine the threshold for genome-wide significance. Haplotypes were calculated with the HAPLOTYPE procedure of SAS/GENETICS, version 9.4 (SAS Institute), for significantly associated X chromosomal SNPs using all 18 genotyped animals.

The GWAS using a mixed linear model revealed a highly significant genome-wide association for eight SNPs on CFAX for the lethal X-linked facial dysmorphia ($P_{raw} = 10^{-22.37}$, $P_{Bonferroni\text{-}corrected} = 10^{-17.37}$) (Fig. 2). These SNPs mapped to a 3.913 Mb region at 17 721 489–21 634 355 bp of CFAX (Table 1, Fig. 3). The affected males were hemizygous for the respective associated allele, obligate female carriers were heterozygous and other females were heterozygous or homozygous for the alternate alleles. Only one unaffected male was hemizygous for the associated allele. For this unaffected male, a recombination event was not obvious when using haplotype data of the region at 16–20 Mb on CFAX. All eight significantly associated SNPs on CFAX were in complete LD with pairwise $r^2 = 1.0$ (Fig. 3). These SNPs constituted significantly associated haplotypes with T–T–C–A–G–A–G–A associated with lethal X–linked facial dysmorphia and C–C–T–G–A–G–C–C associated with unaffected puppies (Table 2). Single SNPs and haplotypes containing the significantly associated SNPs from the proximal X chromosomal region were consistent with an X-linked recessive inheritance for this condition.

In the close neighborhood upstream of the highly associated region at 15 991 623–16 079 689 bp (CanFam3.1), we identified the gene *ribosomal protein S6 kinase A3* (*RPS6KA3*) that causes Coffin–Lowry syndrome (CLS) in humans (Trivier *et al.* 1996) and mice (Marques Pereira *et al.* 2009). In humans, CLS is characterized by moderate-to-severe intellectual disability and typical facial, hand and skeletal malformations (Jacquot *et al.* 2002). Characteristic craniofacial features in humans include prominent forehead and supraorbital ridges, widely spaced eyes with down-slanted palpebral fissures, thick lips and a broad nose with a thick nose septum, anteverted nares, wide mouth and microcephaly or macrocephaly (Jacquot *et al.* 2002; Marques Pereira *et al.* 2009). Patients also often display pectus carinatum or excavatum, kyphosis and scoliosis (Marques Pereira *et al.* 2009). CLS phenotypes in the mouse also include craniofacial dysplasia with decreased cranium length and dental abnormalities (Laugel-Haushalter *et al.* 2014). *RPS6KA3* encodes ribosomal S6 kinase (RSK2), a growth-factor-regulated protein kinase. More than 140 different *RPS6KA3* mutations causing CLS have been identified in human, with most of them being sporadic (Jacquot *et al.* 1998). In a *RSK2* knockout mouse model, lack of phosphorylation of the transcription factor ATF4 by RSK2 was identified to be the cause of the skeletal abnormalities via impaired osteoblast function (Marques Pereira *et al.* 2009). This makes *RPS6KA3* a compelling candidate gene for X-linked facial dysmorphia with severe growth retardation in Labrador Retrievers. Despite a high density of informative markers for CFAX and the particular critical region with its down- and upstream neighborhood, we were not able to identify significantly associated SNPs or haplotypes spanning the candidate gene *RPS6KA3*. In the GWAS, we had seven informative SNPs at 15 936 426–16 250 160 bp (CanFam3.1) with a mean MAF of 0.22 surrounding the candidate *RPS6KA3*. One possible reason

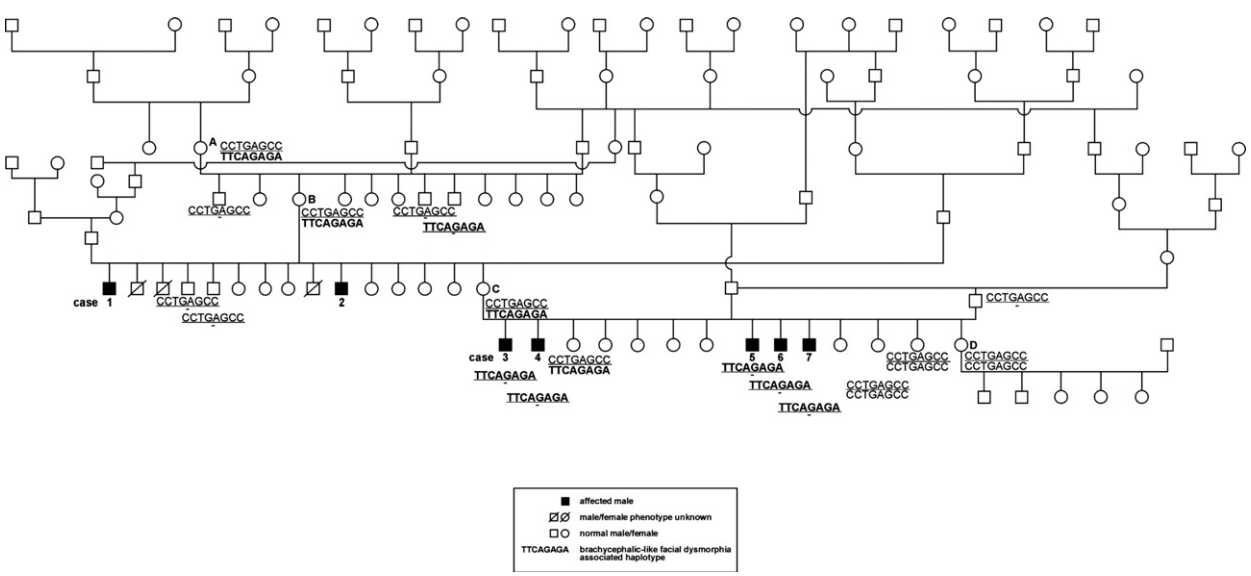

Figure 1 Pedigree for Labrador Retrievers affected by brachycephalic-like facial dysmorphia. Filled symbols mark the brachycephalic-like facial dysmorphia-affected puppies. Haplotypes of the genotyped individuals are given whereby the brachycephalic-like facial dysmorphia-associated haplotype is in bold.

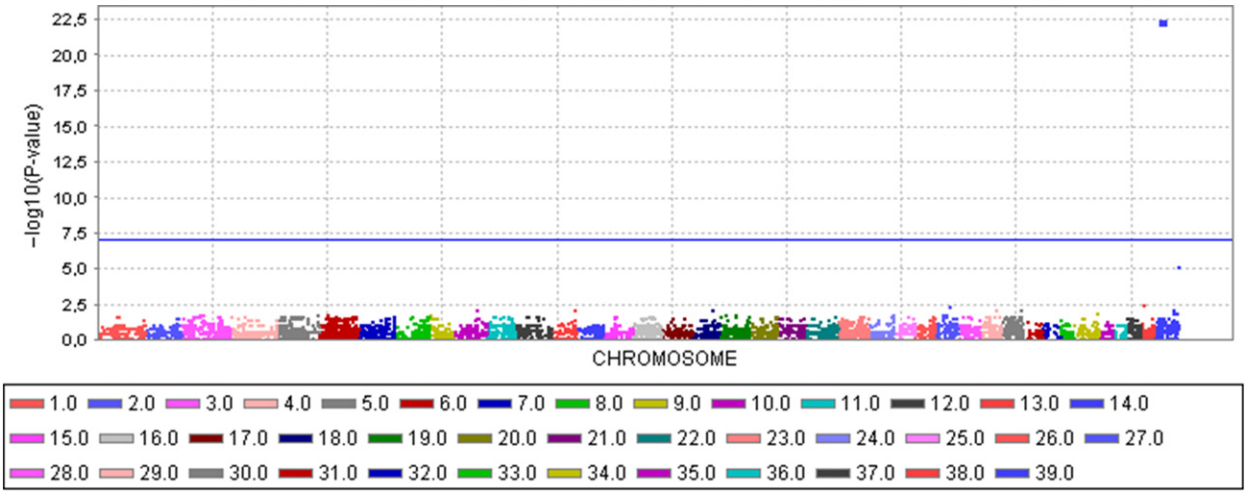

Figure 2 Manhattan plot of the GWAS for brachycephalic-like facial dysmorphia phenotype in Labrador Retrievers using a mixed linear model analysis. The genome-wide $-\log_{10}P$-value for each SNP is plotted against its position on each chromosome. The color codes for the chromosomes are given below the plot. The threshold for genome-wide significance at a $-\log_{10}P$-value of 6.3 is displayed by a blue line. Autosomes are numbered from 1 to 38 and the X chromosome is 39.

SNP position CanFam2.0	SNP position CanFam3.1	SNP-ID	$-\log_{10}P_{raw}$	$-\log_{10}P_{Bon}$	Associated allele
17 721 489	17 770 381	BICF2G630535513	24.35	19.36	T
17 969 316	18 018 434	BICF2G630535486	24.35	19.36	T
18 045 912	18 095 030	BICF2P667349	24.35	19.36	C
21 376 789	21 425 515	BICF2P1036462	24.35	19.36	A
21 386 894	21 435 620	BICF2P1364398	24.35	19.36	G
21 393 161	21 441 887	BICF2P1300711	24.35	19.36	A
21 402 979	21 451 705	BICF2P341503	24.35	19.36	G
21 634 355	21 683 089	BICF2G630535042	24.35	19.36	A

Table 1 Significantly associated SNPs from the GWAS for lethal brachycephalic-like facial dysmorphia, their positions on the X chromosome and raw and Bonferroni-corrected $-\log_{10}P$-values ($-\log_{10}P_{raw}$ and $-\log_{10}P_{Bon}$) as well as the associated alleles.

for this outcome may be recombination events in this region. Another reason may be that associated variants may be more family-specific for this inborn malformation and thus not captured in the SNP content of the canine high-density BeadChip. In addition, it is not clear whether breeders had noticed this condition in generations before the present cases were reported, and the condition may have segregated since longer time.

A further possible candidate gene within the associated region may be spermine synthase (*SMS*) at 17 748 248–17 803 342 bp (CanFam3.1) on CFAX. Snyder–Robinson syndrome was associated with SMS in human. Human patients show diminished muscle mass, osteoporosis, kyphoscoliosis, facial dysmorphism, long great toes, myoclonic or myoclonic-like seizures and renal abnormalities (Peron et al. 2013). Phenotypic findings in affected Labrador puppies investigated here do not agree well with the human Snyder–Robinson syndrome.

Marker and haplotype analysis revealed that the brachycephalic-like facial dysmorphia-associated haplotype has been present for at least four generations in the pedigree. All affected and carrier animals were traced back to a female breeding dog (dog A, Fig. 1), which is the granddam of the litters with cases 1 and 2. In other progeny of dog A or progeny from female ancestors of dog A, this lethal brachycephalic-like facial dysmorphia was not observed. It is possible that this lethal mutation arose in the germline of dog A. This complies with the large size of the significantly associated haplotype having arisen four to five generations ago.

The three genotyped female offspring of the female carrier (dog C) had haplotypes on both X chromosomes not associated with this lethal condition and one of them (dog D) was bred from. In this litter, the two males were normal and the females as well.

The condition herein presented is phenotypically and genetically different from breed-specific canine brachycephaly in French Bulldog, Pug, Pekingese and Boxer. An X-linked inheritance was not obvious for breed-specific canine brachycephaly, nor was it lethal (Haworth *et al*.

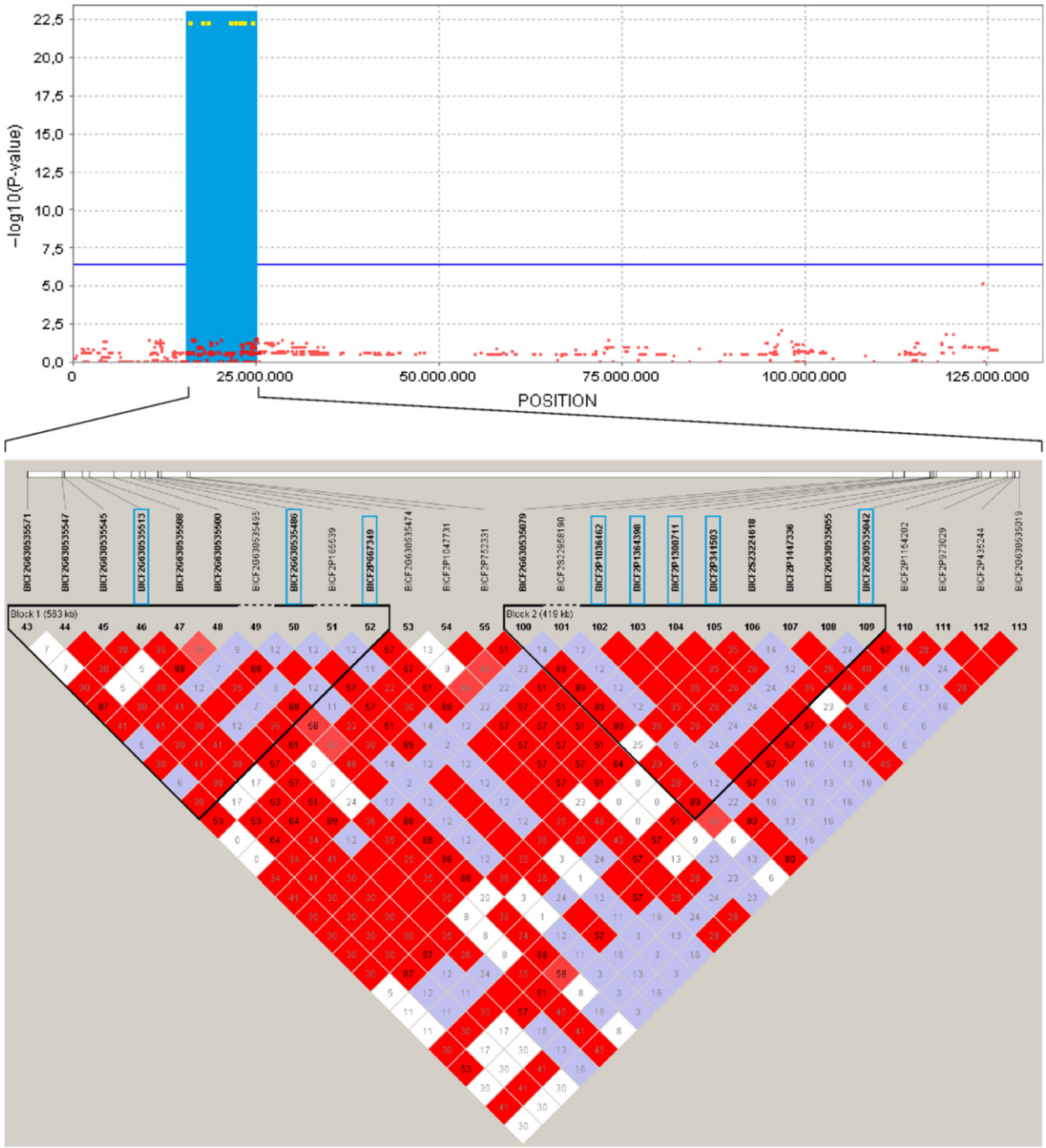

Figure 3 Genome-wide association for brachycephalic-like facial dysmorphia phenotype in Labrador Retrievers on dog chromosome X (CFAX). The $-\log_{10}P$-values of all eight SNPs with their chromosomal position on CFAX, their haplotype structure and LD are shown at 17.5–18.2 and 21.2–21.8 Mb (CanFam2.0). The horizontal line at $-\log_{10}P = 6.3$ indicates the threshold for genome-wide significance. The figure below the Manhattan plot for CFAX displays the region from 17 482 860 to 18 184 502 bp and from 21 214 670 to 21 759 135 bp (CanFam2.0) using Hedrige's multiallelic *D*, which represents the degree of LD between each pair of SNPs. Red fields display LOD ≥ 2 ($D' = 1$), shades of red show the same LOD with $D' < 1$. White and blue fields display LOD < 2 with $D' < 1$ and $D' = 1$. Numbers within fields give r^2 values for the SNPs. Fields with squared correlations at 1.0 are left blank. Genome-wide significantly associated SNPs are located in block 1 (BICF2G630535513, BICF2G630535486 and BICF2P667349) and block 2 (BICF2P1036462, BICF2P1364398, BICF2P1300711, BICF2P341503 and BICF2G630535042). The eight-SNP haplotype associated with lethal X-linked facial dysmorphia was in complete LD with all other SNPs within blocks 1 and 2.

2001; Haworth *et al.* 2007; Bannasch *et al.* 2010; Boyko *et al.* 2010; Quilez *et al.* 2011; Schoenebeck *et al.* 2012; Schoenebeck & Ostrander 2013; Marchant *et al.* 2017). Only an X chromosomal region at 105 274 087–106 866 624 bp was found in a previous study to be associated with skull length and body size (Boyko *et al.*

Table 2 Haplotype-trait association using all eight significantly associated SNPs for lethal brachycephalic-like facial dysmorphia phenotype.

Haplotype	Trait association	χ^2	*P*-Value
T–T–C–A–G–A–G–A	Affected	17.3077	0.000032
C–C–T–G–A–G–C–C	Unaffected	17.3077	0.000032

2010). The brachycephalic-like facial dysmorphia in these Labrador Retriever puppies is to the best knowledge of the authors the only case in dogs where brachycephalic offspring had mesocephalic parents (Dierks *et al*. 2017). In conclusion, we verified the X-linked inheritance of this case and identified a significantly associated X chromosomal region and *RPS6KA3* as a candidate gene. Further work is warranted to unravel the responsible mutation using whole-genome sequencing data.

Acknowledgements

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Availability of data

The SNP genotype data are available in the Figshare Repository (https://figshare.com/articles/Genome-wide_association_study_for_lethal_brachycephalic-like_facial_dysmorphia_in_Labradar_retrievers/9929714).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Figure S1 A puppy with brachycephalic-like facial dysmorphia (a) and a normal puppy about the same age from the same kennel (b).