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MLPH Genotype—Melanin Phenotype Correlation in Dilute Dogs

M. Welle, U. Philipp, S. Rüfenacht, P. Roosje, M. Scharfenstein, E. Schütz, B. Brenig, M. Linek, L. Mecklenburg, P. Grest, M. Drögemüller, B. Haase, T. Leeb, and C. Drögemüller

From the Institute of Genetics, Vetsuisse Faculty, University of Berne, Bremgartenstrasse 109a, 3001 Berne, Switzerland (Haase, Leeb, M. Drögemüller, and C. Drögemüller); the Institute of Animal Breeding and Genetics, University of Veterinary Medicine Hannover, Bünteweg 17p, Hannover, Germany (Philipp); the Institute of Animal Pathology, Vetsuisse Faculty, University of Berne, Länggassstr. 122, Berne, Switzerland (Welle); the Division of Clinical Dermatology, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Berne, Länggassstr. 128, Berne, Switzerland (Rüfenacht and Roosje); the Institute of Veterinary Medicine, University of Göttingen, Burckhardtweg 2, Göttingen, Germany (Scharfenstein, Schütz, and Brenig); the Tierärztliche Spezialisten, Rodigallee 85/Holstenhofweg, Hamburg, Germany (Linek); the Consultant Veterinary Pathology, Bellevue 23, Hamburg, Germany (Mecklenburg); the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zürich, Winterthurerstr. 268, Zürich, Switzerland (Grest); and the Dermfocus, Vetsuisse Faculty, University of Berne, Berne, Switzerland (Haase, Leeb, C. Drögemüller, Welle, Rüfenacht, and Roosje).

Address correspondence to Prof. Dr. Tosso Leeb, Institute of Genetics, Vetsuisse Faculty, University of Berne, Bremgartenstrasse 109a, 3001 Berne, Switzerland, or e-mail: tosso.leeb@itz.unibe.ch.

Abstract

Coat color dilution in dogs is a specific pigmentation phenotype caused by a defective transport of melanosomes leading to large clumps of pigment. It is inherited as a Mendelian autosomal recessive trait and may be accompanied by hair loss, the so-called color dilution alopecia (CDA), or black hair follicular dysplasia (BHFD). We previously identified the noncoding c.-22G>A transition in the melanophilin gene (*MLPH*) as a candidate causative mutation for the dilute phenotype. We have now extended our study and genotyped 935 dogs from 20 breeds segregating for dilute coat color. The dilute-associated A allele segregates in many different breeds suggesting an old mutation event. We also investigated skin biopsies of dogs suspected of having either CDA or BHFD, and our data clearly indicate that the dilute mutation is required but not sufficient to develop clinical signs of the disease. The risk to develop CDA/BHFD seems to be breed specific. Interestingly, 22 out of 29 dogs with clinical signs of CDA/BHFD have clumped melanin in the epidermis, the follicular epithelium, and the hair shafts, whereas in dilute dogs without clinical disease, clumped melanin is only found in the follicular epithelium and the hair shafts but not in the epidermis.

Key words: *black hair follicular dysplasia, coat color, color dilution alopecia, dog, melanophilin*

Dogs with dilute coat color are known in many breeds. In dilute dogs, the eumelanin- or phaeomelanin-pigmented skin appears paler and is denoted breed specific, foe example, blue, gray, Isabella, fawn, silver, or pale brown (Schmutz and Berryere 2007). As the change in phaeomelanin is not as dramatic as the eumelanin dilution, red-colored dogs are sometimes difficult to detect as dilute. Coat color dilution (*d*) is inherited as a Mendelian autosomal recessive trait in various dog breeds (Schmutz et al. 1998). Coat color dilution is characterized by a defective transport of melanosomes within follicular melanocytes, which is mainly regulated by 3 interacting proteins (MLPH, MYO5A, and RAB27A) (Barral and Seabra 2004; Hume et al. 2006; Hume et al. 2007). The dilution phenotype occurs in different mammalian species, and causative mutations within the melanophilin gene (*MLPH*) have been identified in, for example, human, mouse, and cat (Matesic et al. 2001; Ménasché et al. 2003; Ishida et al. 2006). In a previous study, we applied a candidate gene approach and showed that dilute dogs from different breeds share a common approximately 10-kb haplotype block at the 5' end of the *MLPH* gene. Within this shared haplotype block, a noncoding single nucleotide polymorphism (SNP) at the splice donor of exon 1 (c.-22G>A) represents a candidate causal mutation for coat color dilution in 7 dog breeds. The *MLPH* mRNA expression in skin biopsies of dilute beagles carrying the mutant A allele was lower than in beagles carrying the wild-type G allele (Philipp, Hamann, et al. 2005; Philipp, Quignon, et al. 2005; Drögemüller et al. 2007).

Coat color dilution has been described as a predisposing risk factor for certain forms of hair loss in dogs (Mecklenburg 2006). Both, color dilution alopecia (CDA; also known as color mutant alopecia) and black hair follicular dysplasia (BHFD), also known as dark hair follicular dysplasia (Selmanowitz et al. 1972), are primarily noninflammatory forms of hair loss that occur in various dog breeds. CDA is associated with a dilute coat color (Laukner 1998), and hair loss is usually most severe on the dorsal trunk. BHFD occurs in the pigmented coat areas of spotted dogs, for example, in the Large Munsterlander (Schmutz et al. 1998). Some authors consider both diseases to be etiologically identical (Carlotti 1990). BHFD is usually clinically noted within the first weeks of age and comprises fracture of hair shafts in dark coated regions, resulting in partial alopecia and scaling. First clinical signs of CDA are usually noticed between 3 and 12 months of age, rarely later in life, and lesions are usually slowly progressive with age. Affected dogs are prone to secondary pyoderma. With regard to histopathology, CDA is identical to BHFD (Gross et al. 2005, Mecklenburg 2006). Affected skin reveals large clumps of melanin within melanocytes in the hair matrix, the outer root sheath of the hair follicle, and within the hair shaft. The affected hair shafts frequently break within the hair canal resulting in a more or less distorted follicular infundibulum, which is often plugged with keratin, fragmented hairs, and large irregular clumps of melanin. The amount of clumped melanin is variable among breeds and individuals and so is the expressivity of clinical disease (Hargis et al. 1991; Mecklenburg 2006). Some dogs with exactly the same histological findings in the hair follicles have no signs of alopecia, whereas others may have complete hair loss. About 25% of the gray/blue dogs of both sexes show clinical symptoms, whereas the others do not develop alopecia. In addition, not all dogs that have symptoms develop them at a similar age of onset or with similar severity (Schmutz and Berryere 2007). Obviously, some dog breeds, for example, the Large Munsterlander (von Bomhard et al. 2006), develop more easily clinical symptoms than other breeds, such as the Weimaraner, where if symptoms occur at all, they are less pronounced (Laffort-Dassot et al. 2002; Schmutz and Berryere 2007). Unfortunately, no comprehensive study on the breed distribution of CDA or BHFD has been published. A possible influence of other genes besides *MLPH* influencing the expressivity of clinical disease is under debate, and recently the canine *RAB27A* gene was analyzed as possible candidate gene. However, no indication for associated nucleotide polymorphisms in the coding region of *RAB27A* was found (Schmutz and Berryere 2007).

We now conducted an extensive screening experiment to survey in which breeds the *MLPH* c.-22G>A mutation occurs and for which of the various coat colors in different breeds this mutation might be causative. We also investigated the *MLPH* genotypes and the histopathological findings in skin biopsies of 45 dogs suspected of having either CDA or BHFD in order to develop an improved phenotypic classification of CDA and a better understanding for additional disease-promoting factors apart from coat color dilution.

Materials and Methods

MLPH c.-22G>A SNP Genotyping

DNA prepared from ethylenediaminetetraacetic acid (EDTA)–stabilized blood samples of 935 purebred individuals was available for genotyping. These dogs were from 20 breeds, in which the dilute phenotype occurs (Table 1). The coat color phenotype of the dogs was recorded based on photographs and/or pedigree certificates as described (Philipp, Hamann, et al. 2005). Additionally, paraffin-embedded skin biopsies of 45 dogs with melanin aggregates within the hair follicles were available. The skin biopsies were from 40 purebred dogs and from 5 mongrels (Table 2). DNA was isolated from the skin biopsies using the DNeasy Blood and Tissue Kit following the manufacturer's instructions (Qiagen, Hombrechtikon, Switzerland).

Polymerase chain reaction (PCR) for amplifying a 312-bp fragment containing canine *MLPH* exon 1 using the primers MLPH_157395_F (5′-CCTTCCTTCCCCTGTAGGAC-3′) and MLPH_157706_R (5′-GCCTAAAAT-GAGCTCCCTGA-3′) and sequence reactions were carried out as described before (Drögemüller et al. 2007). Sequence data were analyzed with Sequencher 4.8 (GeneCodes, Ann Arbor, MI) to derive the genotypes at the *MLPH* c.-22G>A polymorphism. Alternatively, the PCR reaction was carried out using the same primers in a LightCycler where a melting point analysis was performed after amplification using the sensor oligonucleotides Dil(AiO)E1Pw (5′-ROX-GAA AGG AGC CGG TGA GTG CA-PHO-3'), Dil(AiO)E1Pm (5′-Cy5.5-GAA AGG AGC CAG TGA GTG CAG-PHO-3′), and the anchor oligonucleotide Dil(AiO)Ex1A (5′-CCA GGG CCT GCC CGC CCC G-fluoresceine-3′). The mutation is characterized by a sensor probe half melting temperature of 60 °C (Dil(AiO)E1Pw) or 67 °C (Dil(AiO)E1Pm) as compared with 66 °C or 61 °C for the wild-type allele, respectively.

Histopathology

One to five routinely processed skin biopsies stained with hematoxylin and eosin from a total of 45 dogs were examined in this study. They were selected from archival material of the Institute for Animal Pathology, Vetsuisse Faculty, University of Berne, Berne, Switzerland (35 cases), and the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland (10 cases). In all histological reports of these biopsies, clumped melanin in the hair follicle and hair shafts had been described. For this study, a blinded histological examination of the biopsies was performed by one of the authors.

Results and Discussion

In a previous study, we have identified the c.-22G>A transition at the last nucleotide of the 5′-untranslated first exon of the *MLPH* gene as a candidate causative mutation for the dilute phenotype (Drögemüller et al. 2007). We have

Table 1. Genotype frequencies of the *MLPH* c.-22G>A SNP in 935 dogs with Coat color records

Breed	Coat color	No. of animals	Exon 1 SNP (c.-22G>A)		
			A/A	A/G	G/G
German pinscher[a]	Dilute	28	28		
	Wild type	363		131	232
Doberman pinscher[a]	Dilute	26	26		
	Wild type	208		77	131
Rhodesian ridgeback[a]	Dilute	8	8		
	Wild type	112		35	77
Whippet	Dilute	11	11		
	Wild type	64		38	26
Australian shepherd[a]	Dilute	3	3		
	Wild type	21		8	13
Briard[a]	Dilute	6	6		
	Wild type	12		6	6
Bolonka Zwetna[a]	Dilute	3	3		
	Wild type	11		9	2
French bulldog	Dilute	2	2		
	Wild type	11		6	5
Great Dane	Dilute	3	3		
	Wild type	5		2	3
Chihuahua	Dilute	4	4		
	Wild type	3		3	
Beagle[a]	Dilute	2	2		
	Wild type	5		4	1
Large Munsterlander[a]	Dilute	2	2		
	Wild type	4		4	
Newfoundland	Dilute	2	2		
	Wild type	2		1	1
Hovawart	Dilute	2	2		
	Wild type	2		2	
Miniature pinscher	Dilute	3	3		
Border collie	Dilute	2	2		
Slovakian rough haired pointer	Dilute	2	2		
American Staffordshire terrier	Dilute	1	1		
Italian greyhound	Dilute	1	1		
Jack Russel terrier	Dilute	1	1		
Total		935	112	326	497

[a] Perfect cosegregation of the A allele with the dilute coat color was also observed in informative families of these breeds.

now extended our study and genotyped 935 dogs with recorded coat color from 20 different dog breeds at this SNP marker (Table 1). In 9 of the 20 dog breeds, informative 2-generation families were available, and in these families the A allele showed perfect cosegregation with the dilute phenotype. Furthermore, we observed perfect association of the dilute phenotype with the c.-22G>A polymorphism in all 20 different breeds, and each of the 112 dilute colored dogs in our study was homozygous for the A allele at the c.-22G>A polymorphism (Table 1). We still cannot formally exclude the possibility that c.-22G>A represents just a neutral polymorphism in linkage disequilibrium with the causative mutation until the complete associated haplotype block has been fully characterized in the dilute animals. However, the new genotype data from the extended large cohort are in agreement with our previous findings and support the hypothesis that c.-22G>A is indeed the causative mutation. The wide breed distribution across diverse types of dogs implies an old mutation event predating the creation of modern dog breeds.

Dilute colored dogs are assumed to be predisposed to develop hair loss in the form of CDA or BHFD. In order to get a more accurate phenotypic classification, we histologically examined archived biopsies of 45 unrelated dogs with an original histological finding of clumped melanin within the hair follicle and clinical hair loss. On re-evaluation in 29 of the 45 cases, the clinical findings were compatible with the described findings in CDA or BHFD (Gross et al. 2005). In all 45 cases, clumped melanin of variable size in the hair shaft and the outer root sheath of the follicle was present. Some hair shafts were fractured or attenuated. The sometimes dilated infunbibula contained variable amounts of keratin, hair fragments, and melanin clumps. A rather high percentage of hair follicles was in telogen, and perifollicular pigmentary incontinence of variable degree was present. In some of the biopsies, additional findings

Table 2. Histological phenotypes of 38 biopsies from dogs, which were homozygous A/A at *MLPH* c.-22G>A and originally suspected of being affected with CDA or BHFD

Breed	No. of animals	Clinically CDA/BHFD and clumped melanin in epidermis and follicle	Clinically CDA/BHFD and clumped melanin only in follicle	Clinically other forms of alopecia and clumped melanin only in follicle
Doberman pinscher	7	4	3	
Yorkshire terrier	7	5	2	
German pinscher	2			2
Chihuahua	5	3	1	1
Rhodesian ridgeback	2	2		
Staffordshire bull terrier	1	1		
Bernese mountain dog	1			1
Giant schnauzer	1			1
Great Dane	1	1		
Italian greyhound	1	1		
Miniature pinscher	1			1
Poodle	1			1
Prague Ratter	1		1	
Tibetan terrier	1			1
Weimaraner	1	1		
Mongrels	5	4		1
Total	38	22 (58%)	7 (18%)	9 (24%)

compatible with a secondary pyoderma were seen. The 45 biopsies were also genotyped at the *MLPH* c.-22G>A SNP. The coat color phenotypes of the 45 dogs were not available. A total of 38 out of 45 dogs were homozygous A/A for the dilute-associated allele. Two dogs with aggregated melanin within the hair follicles and hair shafts were heterozygous A/G, and 5 dogs were homozygous G/G. Surprisingly, some clumped melanin was present in the outer root sheath of the follicles in these 7 dogs. However, clumping of melanin is not only specific for CDA and BHFD but also occurs in hair follicle dystrophy as a consequence of preterminal catagen induction (Hendrix et al. 2005). All remaining 38 homozygous A/A cases showed clumped melanin in the hair follicles. In 22 dogs (58%), where the clinical anamnesis was exactly compatible with the criteria of CDA or BHFD, clumped melanin was visible in the epidermis (Figure 1a). In 7 dogs (18%), which also showed typical clinical symptoms of CDA or BHFD, the clumped melanin could only be seen in the hair follicles, and we consider these dogs to be most likely affected with CDA or BHFD (Figure 1b). In the remaining 9 dogs (24%), where the clumped melanin was also apparently restricted to the hair follicles, the clinical symptoms were not compatible with CDA or BHFD after careful re-evaluation of the available anamneses (Figure 1c). In some of these dogs, the re-evaluation resulted in the diagnosis of other well-known hair loss phenotypes, such as, for example, seasonal flank alopecia or endocrine disorders.

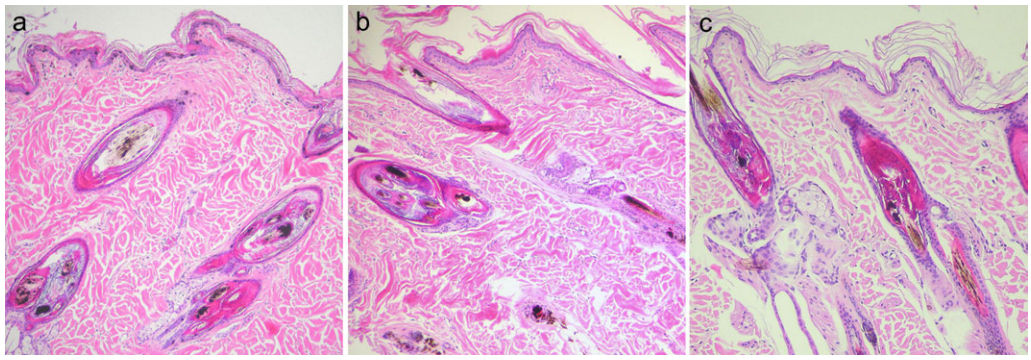

Figure 1. Representative histological phenotypes of dogs, which were homozygous A/A at *MLPH* c.-22G>A and suspected of being affected with CDA or BHFD. Biopsies from dogs with (**a**) confirmed clinical symptoms of CDA/BHFD and clumped melanin in epidermis, hair follicles, and hair shafts; (**b**) clinical symptoms compatible with CDA/BHFD and clumped melanin only in the hair follicle; and (**c**) clinical symptoms different from accepted CDA/BHFD criteria and clumped melanin restricted to the hair follicle. Note that melanin clumping in the hair follicle is similar in all 3 cases, and the definitive diagnosis of CDA/BHFD can only be made based on additional clinical findings. Hematoxylin and eosin ×200.

From the detailed histological analysis of the 38 biopsies from homozygous A/A dogs, we suspect that the current histopathological diagnostic criterium of clumped melanin in the hair follicle does not allow an unambiguous phenotype classification of clinically suspected CDA or BHFD cases. The histhopathological findings have to be evaluated in light of the clinical symptoms to allow a precise and definitive diagnosis of CDA or BHFD. In about a quarter of the dogs that had originally been diagnosed with CDA or BHFD, the hair loss is apparently caused by a different pathological mechanism, and therefore, these dogs should not be considered to be genuinely affected by CDA or BHFD. According to our findings, clumped melanin in the epidermis is a very strong indicator of true CDA or BHFD.

In conclusion, we found perfect association of the *MLPH* c.-22G>A SNP with dilute coat color in more than 900 dogs supporting the hypothesis that this polymorphism is indeed the causative mutation. The wide breed distribution of the mutant *MLPH* c.-22G>A allele suggests an old mutation event. Although our data clearly indicate that the *MLPH* mutation increases the risk for CDA/BHFD, there seem to be additional modifying factors. A characteristic feature of most CDA/BHFD–affected dogs is the presence of clumped melanin in the epidermis. In some breeds, such as the pinscher breeds and the Rhodesian ridgebacks, the reported coat quality of dilute dogs ranges from normal to severely CDA affected. Therefore, these breeds offer the chance to search for modifier genes, which influence the risk of developing CDA/BHFD in dogs with dilute coat color.

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