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MC1R Studies in Dogs With Melanistic Mask or Brindle Patterns

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Abstract

Black mask is a characteristic pattern in which red, yellow, tan, fawn, or brindle dogs exhibit a melanistic muzzle which may extend up onto the ears. Melanistic mask is inherited in several breeds as an autosomal dominant trait, and appears to be a fixed trait in a few breeds of dogs. A *MC1R* nonsense mutation, R306ter, has been shown to cause a completely red or yellow coat color in certain breeds such as Irish setters, yellow Labrador retrievers, and golden retrievers. The amino acid sequence for the melanocortin receptor 1 gene (*MC1R*) was examined in 17 dogs with melanistic masks from seven breeds, 19 dogs without melanistic masks, and 7 dogs in which their coat color made the mask difficult to distinguish. We also examined nine brindle dogs of four breeds, including three dogs who also had a black mask. No consistent amino acid change was observed in the brindle dogs. All dogs with a melanistic mask had at least one copy of a valine substitution for methionine at amino acid 264 (M264V) and none were homozygous for the premature stop codon (R306ter). These results suggest that black mask, but not brindle, is caused by a specific *MC1R* allele.

Coat color in dogs has been of interest to breeders since breed registries began. A few breeds of dogs have a brindle, tan, yellow, fawn, or other pale coat color of pheomelanin pigment over most of their body, but may have a black, brown, or gray mask over their muzzle (Figure 1). This black muzzle sometimes extends up over their ears. The pale coat color is thought to represent a specific pigment type, pheomelanin, chemically and ultrastructurally distinct from eumelanin, which is black, brown, or gray. Breeds that sometimes or always have such a black mask include the Akita, bullmastiff, boxer, German shepherd, Great Dane, greyhound, keeshond, Leonberger, mastiff, Pekingese, pug, Rhodesian ridgeback, sloughi, Tibetan spaniel, and whippet. Clarence C. Little, who pioneered the study of inheritance of coat colors and patterns in dogs in North America, proposed that a major determinant of pheomelanic coat color and the black mask was allelic variation at the *Extension* (*E*) locus, now known to represent the melanocortin 1 receptor (MC1R). Little (1957) proposed that dogs which were *ee* would produce pheomelanin coat colors such as yellow, gold, apricot, or red and that dogs which were black or brown always had one *E* allele. He further proposed that a dominant allele at the *E* locus, E^M, was responsible for causing dogs to have a black mask and that a single copy of the allele e^{br} was responsible for the brindle pattern. Brindle is a pattern of alternating eumelanin and pheomelanin stripes in dogs which is obvious as stripes in short-haired breeds such as greyhounds, whippets, and Great Danes (Figure 1), but appears more as multicolored hairs on longhaired breeds such as Scottish deerhounds and Bouviers.

In other mammals, major determinants of the balance between eumelanin and pheomelanin synthesis are allelic variation at the *MC1R* locus and the *Agouti* locus. *MC1R* encodes a seven transmembrane-spanning receptor that, when active, causes hair follicle melanocytes to produce eumelanin instead of pheomelanin. *Agouti* protein is a paracrine signaling molecule secreted by specialized cells adjacent to hair follicle melanocytes that inhibits the *MC1R*. Thus *MC1R* loss of function or *Agouti* gain of function favors production of pheomelanin, while *MC1R* gain of function or *Agouti* loss of function favors production of eumelanin; in general, *MC1R* is epistatic to *Agouti*.

Newton et al. (2000) and Everts et al. (2000) described a premature stop codon, R306ter, in the dog melanocortin receptor 1 (*MC1R*) gene that was present in the homozygous state in dogs with red or yellow coat color, such as red Irish

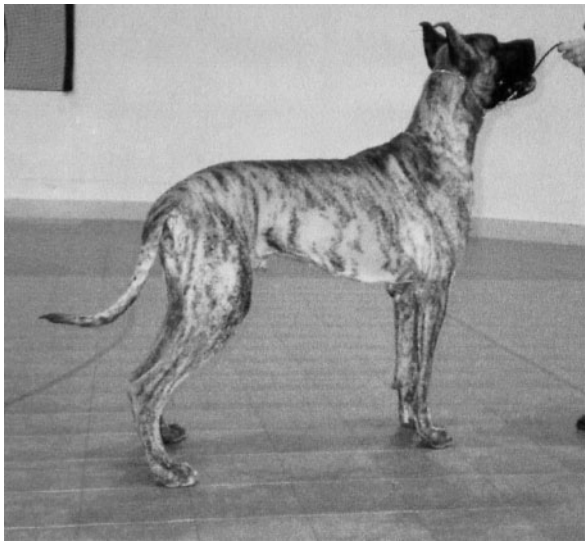

Figure 1. Photograph of a Great Dane named Banjo who has a melanistic or black mask and a brindle body pattern.

setters or yellow Labrador retrievers. This established that *MC1R* was indeed the same as the *Extension* locus, as has been shown to be the case in many other species such as cattle (Joerg et al. 1996; Klungland et al. 1995), horse (Marklund et al. 1996), and pig (Kijas et al. 1998). This work was extended by Schmutz et al. (2002) to demonstrate the interaction of these two *MC1R* alleles (*E* and *e*) with *TYRP1* alleles, causing brown coat color and/or nose and pad color in dogs.

In this study we attempted to determine if an *MC1R* allele could be found which was associated with the black mask pattern or the brindle pattern. We therefore examined the DNA sequence of *MC1R* in several dogs of various breeds with black mask and/or brindle pattern.

Materials and Methods

Animals

DNA was obtained from several dogs, using cheek swab brushes (Epicentre, Madison, WI) or Cytobrush Plus GT cervical brushes (Medscand Medical, Malmö, Sweden). Coat color was recorded by the veterinarian and/or owner or by us if we took the sample. Nose color was also recorded in some cases. DNA samples from a litter of Great Dane pups which segregated for brindle were also obtained.

MC1R Sequencing

The primers D and E from Newton et al. (2000) were used to amplify the complete coding sequence. Genomic samples were sufficient since *MC1R* is composed of a single exon. Samples were prepared using the Concert Rapid PCR Purification System (GibcoBRL) and sequenced with an ABI sequencer at the Plant Biotechnology Institute in Saskatoon, Saskatchewan.

Genotyping

The V264M substitution causes an *Nla*III restriction site to be formed with the Met allele. A new forward primer was designed to create a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) test.

Forward: CGCTGCCACACTCACTATCCTGC

Reverse (primer G from Newton et al. 2000): CTGCCCAGCACCCTGGCCTC

Digestion with *Nla*III produced a cut site in the allele containing A to produce a band of 194 and 76 bp.

The premature stop codon at amino acid 306 (Newton et al. 2000) or the *e* allele was detected using the reverse primer G and the purposeful mismatch primer designed for this purpose, CATCTACGCCTTCCGCAGCCAGGAGCGC (Schmutz et al. 2002), and digestion of the product with *Eco*47III. *ZuBeCa6* (Ladon et al. 1998) was used to genotype the Great Dane family, since this microsatellite marker was linked to *MC1R* (Schmutz et al. 2001).

Results

The complete amino acid sequence was obtained from 11 dogs. In addition to the DNA variants previously reported by Newton et al. (2000), we detected an A to G transition in the first position of codon 264 which resulted in a methionine to valine change in several dogs with melanistic facial masks. A G to A transition in the first position of codon 205, which resulted in a valine to methionine change, was detected in a single dog. Variants reported previously (Newton et al. 2000) were also observed (not shown), but did not correlate with coat color phenotype.

We developed PCR-based tests for the M264V variant and the R306ter variant and genotyped a series of dogs. All 17 dogs with a melanistic mask were heterozygous or homozygous for V264 (Table 1). All 19 dogs that did not have black masks were homozygous for M264. Seven dogs presented phenotypes that were difficult to classify as to the presence or absence of a melanistic mask, although all were heterozygous or homozygous for V264 (Table 1). The latter class of exceptions (dogs without a black mask who carried V264) included two dogs (Shadow and Dodger) who probably lack melanocytes over most of their body, three dogs of uniform eumelanin coloration—two toy poodles (brown or silver brown) and a black German shepherd—in whom a eumelanin mask would not have been apparent, and two Scottish deerhounds, in whom gray brindling and longer hair obscured a mask pattern. Thus our results are consistent with the hypothesis that the M264V *MC1R* allele causes the melanistic mask trait.

We found no correlation between *MC1R* variation and the brindled phenotype (Table 1). We also had the opportunity to examine a litter of Great Dane pups in which brindle was segregating (Figure 2). Transmission of brindle in

Table 1. *MC1R* genotypes for dogs with black masks and controls at amino acid 264 and 306

				MC1R residue		
Dog	Breed	Coat color	Mask	Nose color	264	306
Talle	Akita	Pale	Black	Black	V/M	
Ginny	Akita	Pale	Black	Black	V/V	R/R
Fritz	Boxer	Red	Black	Black	V/V	R/R
Ernie	Bullmastiff	Red	Black		V/V	
Freddie	Bullmastiff	Fawn	Black		V/V	
Kukka	Bullmastiff	Fawn	Black		V/V	
Rufus	Bullmastiff	Red	Black		V/V	
Jewel	Great Dane	Fawn	Black		V/V	
Banjo	Great Dane	Brindle	Black	Black	V/V	R/R
Doink	Great Dane	Brindle	Gray	Black	V/M	
Lucy	Greyhound	Brindle	Black	Black	V/M	R/R
Loki	Rhodesian ridgeback	Red	Black	Black	V/V	R/R
Lola	Rhodesian ridgeback	Fawn	Black	Black	V/M	R/R
Sisu	Rhodesian ridgeback	Red	Black	Black	V/M	R/R
CJ	Rhodesian ridgeback	Red	Brown	Brown	V/M	R/R
Uconn	German shepherd	Black and tan	Black	Black	V/V	R/R
Jasmine	German shepherd	Black and tan	Black	Black	V/V	
Shadow	Boxer	White	None	Black	V/V	
Dodger	Greyhound	White, black spots	None	Black	V/M	R/R
Tyee	Scottish deerhound	Gray brindle	Gray?		V/V	R/R
Moon	Scottish deerhound	Gray brindle	Gray?		V/V	R/R
Sinder	German shepherd	Black	Black?	Black	V/M	R/R
Flutey	Toy poodle	Brown	Brown?	Brown	V/M	R/R
Lacey	Toy poodle	Silver brown	Brown?	Brown	V/M	R/R
Kennedy	Bouvier	Brindle	None		M/M	R/R
Smokey	Greyhound	Brindle	None		M/M	R/R
Lucky	Greyhound	Black, roan muzzle	None	Black	M/M	R/R
Sophie	Whippet	Fawn and white	None	Black	M/M	R/R
Cela	Crossbred	Brindle	None		M/M	R/X
Taffy	Crossbred	Brindle	None		M/M	R/X
Pepper	English setter	Tricolor	None	Black	M/M	R/R
Jake	Dachshund	Red with shading	None	Black	M/M	R/R
Gideon	Doberman pinscher	Black and tan	None	Black	M/M	
Bella	Doberman pinscher	Black and tan	None	Black	M/M	
TK	Doberman pinscher	Black and tan	None	Black	M/M	
Candy	Doberman pinscher	Brown and tan	None	Brown	M/M	
Devon	Doberman pinscher	Brown and tan	None	Brown	M/M	
Macy	Doberman pinscher	Brown and tan	None	Brown	M/M	
Sam	Cocker spaniel	Buff and white	None	Black	M/M	X/X
Kelsey	Miniature poodle	Apricot	None	Black	M/M	X/X
Chase	Miniature poodle	Apricot	None	Black	M/M	X/X
Lewey	Miniature poodle	Apricot	None	Black	M/M	X/X
Riley	Irish setter	Red	None	Black	M/M	X/X

M, methionine; V, valine; R, arginine; X = stop.

this pedigree is consistent with autosomal dominant inheritance, with the sire, Banjo, being heterozygous. The Great Dane male, Banjo, was found to be homozygous for the valine substitution at amino acid 264 from sequence. He was subsequently bred to a fawn female who also had a mask and all six of the pups had masks (Figure 2), as one would expect from a homozygote. However, only two of the six pups were brindle; confirming that mask and brindle segregate independently from each other. *MC1R* was mapped to dog chromosome 5 (Schmutz et al. 2001), about 6 cM from the microsatellite marker *ZuBeCa6* (Ladon et al. 1998), using the A105T polymorphism. The Great Dane sire, Banjo, was not heterozygous for any other *MC1R* variant, therefore his family was genotyped using *ZuBeCa6*. Allele sizes (Figure 2) show that all of the offspring, two brindle and four nonbrindle, inherited the same paternal *ZuBeCa6* allele, which suggests that *MC1R* variation is not responsible for brindling in this pedigree.

Discussion

All dogs who had a black mask had a valine instead of a methionine at amino acid 264 of *MC1R*. However, not all

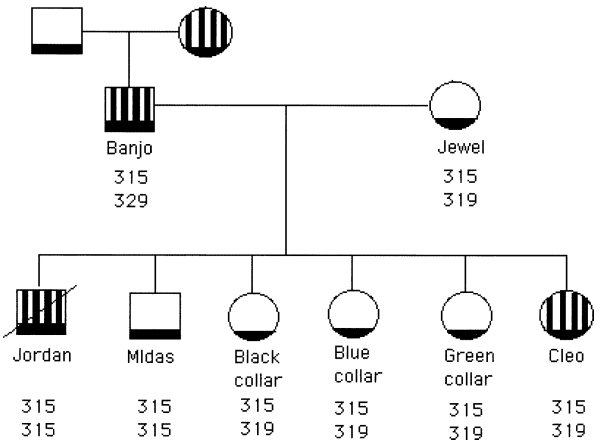

Figure 2. Pedigree of a Great Dane litter sired by a brindle male with black mask and a fawn female with black mask. All dogs had a black mask, but only some were brindle (striped). Genotypes for the *ZuBeCa6* microsatellite which is linked to *MC1R* are shown below each dog.

dogs with this substitution had a black mask. Dogs which are black, such as Sinder (Table 1), cannot show a black mask. Furthermore, dogs which have two "brown" mutations at *TYRP1* would produce brown instead of black eumelanin pigment (Schmutz et al. 2002). Brown dogs, such as the toy poodles Flutey and Lacey, would not show a brown mask on a brown body, as discussed by Willis (1989). Likewise the Rhodesian ridgeback (CJ), which had a brown nose, would have a brown mask that could not be easily distinguished from the red body color. Sometimes masks are also difficult to detect on brindle dogs, since the dark stripes and the width of the stripes vary considerably. Furthermore, in some breeds such as greyhounds, where brindle is often diluted to pale gray stripes on pale yellow, or Scottish deerhounds, which are now all gray brindle, the black mask becomes gray or almost white, as it did in Doink. The pigmentation in the mask responds to other genes affecting the type of eumelanin produced and is therefore a melanistic mask, but not necessarily a black mask.

MC1R variation has been shown to affect hair color variation in a large number of vertebrate species. In most cases, loss-of-function mutations cause a uniform pale and/or pheomelanic coloration, as in humans with carrot-red hair and fair skin (Valverde et al. 1995), yellow Labrador retrievers (Everts et al. 2000; Newton et al. 2000), or very pale Kermode bears, while gain-of-function mutations that encode a constitutively active receptor cause a uniform eumelanic coloration, as in black sheep (Våge et al. 1999), black pigs (Kijas et al. 1998), or dark jaguarundis (Eizirik et al. in press). Coat color phenotypes which show a mixture of pheomelanin and eumelanin in different regions of the body are generally caused by regional expression of *Agouti* or genetically unstable *MC1R* alleles associated with somatic mosaicism. However, an exception is the fox, in which the combination of a dominant *Agouti* allele and a constitutively active *MC1R* cause a mixture of pheomelanin and eumelanin in a patterned distribution (Våge et al. 1997).

In dogs, Little (1957) suggested that the presence of a black mask on an otherwise pheomelanic coat was caused by an *MC1R* allele (known then as *Extension* [E^M]), while Winge (1950), another authority on genetics of dog coat color, suggested that black mask was caused by an *Agouti* allele (Winge used the terminology e^{ma}). Although the dog *Agouti* gene has not yet been characterized, our results are consistent with Little's suggestion, since we observed an association between a specific *MC1R* allele, M264V, and the black mask phenotype. Including black mask and *MC1R* loss of function (*e*) in the same allelic series also implies that the two sequence variants, M264V and R306ter, should never be found in *cis*. Finally, because pheomelanic coat color in breeds with black masks is presumably caused by a gain-of-function *Agouti* allele (described historically as a^y or a^t), red, yellow, or pale animals in these breeds should never be homozygous for R306ter. Both predictions are consistent with our results. Black mask is considered a fixed trait in certain breeds, such as boxers, bullmastiffs, and pugs. Even the white boxer, Shadow, was homozygous for valine at residue 264, although he was unable to produce pigment anywhere and therefore no mask was visible. Our results are unlikely to be explained by chance fixation of an M264V polymorphism, since the association is apparent within greyhounds. Nonetheless, the substitution does not predict an obvious effect on receptor function, and additional pedigree and/or pharmacologic data will be needed to prove convincingly that M264V causes black mask.

Based on biochemical and genetic studies in the mouse, we anticipate that an *MC1R* allele responsible for black mask should increase receptor activity. The predicted location of residue 264 lies at the junction of the sixth transmembrane domain and the third exoloop, and it is possible that M264V could inhibit binding or action of *Agouti* protein, increase the ability of the receptor to couple to adenylate cyclase, or increase the amount of receptor protein at the cell surface. It is also possible that M264V is in linkage disequilibrium with a noncoding sequence variant that causes an increase in steady-state mRNA levels. However, the same amino acid change occurred in dog breeds as evolutionarily divergent as the Akita and the greyhound, which would suggest that this polymorphism had to be very old and remained in linkage disequilibrium with the causative mutation over hundreds of generations.

Regardless of these considerations, a specific *MC1R* allele that causes localized distribution of eumelanin in an otherwise pheomelanic animal is unlikely to be explained by a mutation that causes regional differences in receptor expression, and instead implies the existence of an underlying pattern that affects melanocortin receptor signaling in certain regions of the body. For example, the same developmental mechanisms responsible for regional differences in hair type and hair length (i.e., beards in wirehaired breeds) could also affect the number of melanocytes per hair follicle or the ratio of hair follicle melanocytes to mesenchymal cells that secrete *Agouti* protein. Thus specific

regions of the body might have different thresholds for the switch between eumelanin and pheomelanin synthesis, and germline variation of the *MC1R* sequence might affect the threshold in some but not other regions.

In addition to black mask, Little (1957) also postulated that brindle was part of the *Extension* series (e^{br}), although Winge put brindle in the *Agouti* series. Our results suggest that brindling is not caused by *MC1R* variation, since there was no consistent *MC1R* sequence variant among seven brindle dogs. Three of the brindle dogs were homozygous for M264V, two dogs were heterozygous for A105T, and three dogs were heterozygous (one) or homozygous (two) for P159Q. The latter two variants were present in several dogs genotyped by Newton et al. (2000), none of which were brindle. Most important, in a pedigree where brindle was segregating as a dominant, brindle and nonbrindle animals inherited the same *MC1R* allele from their brindle parent.

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